DOCTORAL THESIS

Speciality
'Océanologie biologique' (École doctoral science de l'environment de l'Ile de France)
& 'Ciências do Mar' (Faculdade de Ciências da Universidade de Lisboa)

Presented by
Miguel José Dias Barbosa da FRADA

To obtain the Doctoral Degree in co-direction between:
'Université Pierre et Marie Curie (Paris VI)' & 'Universidade de Lisboa'

The Haplo-Diplontic Life Cycles of the Haptophytes, with emphasis on the Eco-Physiology of *Emiliania huxleyi*

Defended at Roscoff, the 6 of February 2009

Jury committee :

Dr. Colomban de Vargas, CNRS, Station Biologique de Roscoff	Director
Prof. Mário Cachão, U. Lisboa, FCUL, Departamento de Geologia	Director
Prof. Ana Amorim Ferreira, U. Lisboa, FCUL, Departamento de Biologia Vegetal	Reporter
Dr. Jeremy Young, Museum of Natural History of London	Reporter
Prof. Bernard Kloareg, UPMC, Station Biologique de Roscoff	Examinator
Prof. Bruno de Reviers, Museum d' Histoire Naturelle de Paris	Examinator
Dr. Ian Probert, Station Biologique de Roscoff	Examinator

Ciência.Inovação 2010 Programa Operacional Ciência e Inovação 2010
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

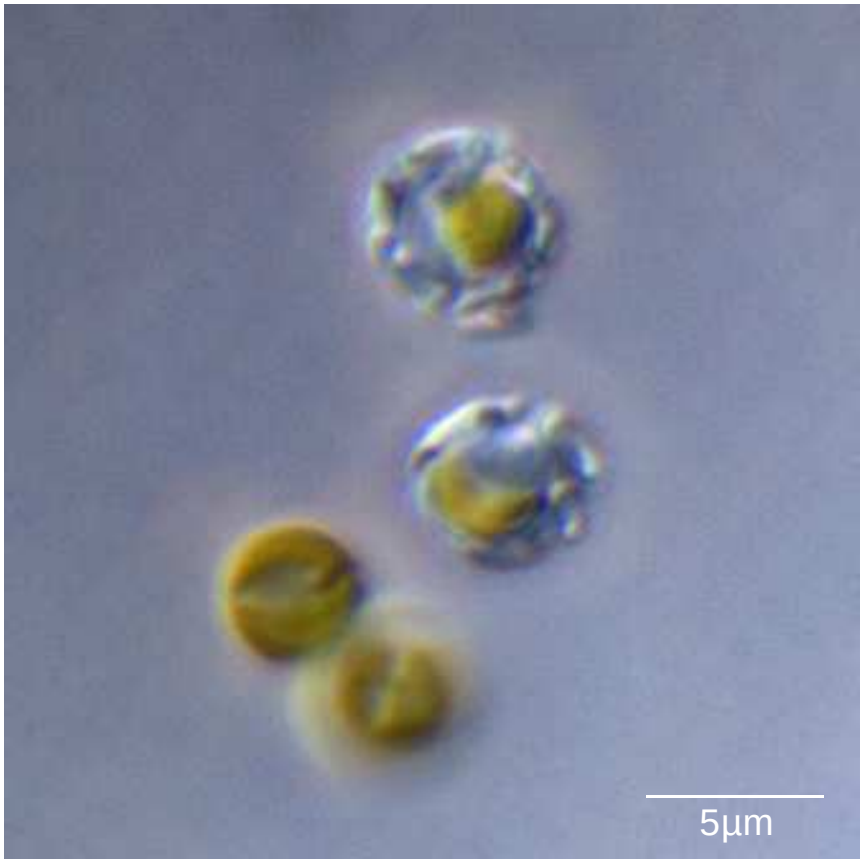
DOCTORAL THESIS

Speciality
'Océanologie biologique' (École doctoral science de l'environment de l'Ile de France)
& 'Ciências do Mar' (Faculdade de Ciências da Universidade de Lisboa)

Presented by
Miguel José Dias Barbosa da FRADA

To obtain the Doctoral Degree in co-direction between:
'Université Pierre et Marie Curie (Paris VI)' & 'Universidade de Lisboa'

The Haplo-Diplontic Life Cycles of the Haptophytes, with major emphazis on the Eco-Physiology of *Emiliania huxleyi*



Legend. Light microscopy image of the two phases of the life cycle of the coccolithophore *Emiliania huxleyi.On top the calcified diploid phase and below the non-calcified flagellated haploid phase.*

Ao meu Pai e à minha Mãe

'to my father and my mother'

Aknowledgements

First of all I would like to express my most deepest and eternal gratitude to two incredible scientists and my scientifical parents: Colomban and Ian. Only their inspired philosophies and overall adivises, support,trust and help have made this work possible...

Agradeço ao Professor Mário…que um dia, em Santa Maria nos Açores, me mostrou pela primeira vez as maravilhas dos nanofósseis. Agradeço-lhe também a disponibilidade e apoio durante o meu trabalho.

Je remercie Fabrice, de m'avoir accueilli la première fois à la Station et de m'avoir montré comment faire du FISH...c'était le début de mon travail de thèse...Merci aussi pour tes conseils.

Je remercie Céline pour son incroyable aide sutout quand je suis arrivé à Roscoff...sans toi je ne serais pas encore inscrit à Paris VI...

I thank the coccolithoviruses experts, Mike and Willie, for their trustfull and friendly collaboration. I thank also all mesocosm 2008 members, including Agnes for her help.

I thank Isabella and Adriana, for their trustfull and unexpected collaboration on the beautiful Pontosphaeraceae life cycles study.

I thank also Jeremy, for the previous collaboration as well as for all the adivses during my work.

Muchas gracias Danieliña por tu ayuda i por tu coraje i détemination durante el 'master 2´.

Je remercie Didier Jollivet et Dominique Le Guen qui m'ont initié á la méthode des allozymes.

Je voudrais remercier toute la famille Plancton....spécialement Daniel, pour m'avoir reçu dans le labo au début de ma thèse, Nathalie pour tous ses conseils, mes copines et collègues de thèse, Aurélie, Elodie, Manon et Sylvie, et aussi Dominique (le super cyto-man et grand footballer du SLK), Florence, Fabienne et Priscillia, pour leur amité et...leur incroyable patience...

Agradeço..je remercie...ARIGATOU...I thank all little EPPOies, António, Mahdi, Sarah, Yoshi, Yurika, Anne-Claire, Johan and Peter...for the friendship and overall enthusiasm!

Je remercie aussi tous les copains et copines roscovites ou ex-roscovites, footballers ou pas (ils se reconnaitront...). Merci pour votre amitié, votre soutien et pour tous les moments de joie et de fête passés à Roscoff pendant ces 3 années!

Un grand merci général à toute la station,le personnel de l'atelier, de la laverie, du service informatique, du service mer et de la cantine...vous faites de cette station un paradis pour faire de la bonne recherche scientifique !

I thank all the members of the jury, for accepting my invitation to evaluate and participate in my final thesis presentation and discussion.

Gostaria de deixar umas palavras também aos 'amigos da minha terra'... especialmente aos açorianos, aos invictos portuenses e aos portistas…mesmo longe, a vossa imagem gravada na minha memória e as recordações felizes dos 'velhos tempos', fortaleceram o meu espírito e motivaram-me para avançar em muitos momentos…

Agradeço também a todos os membros da minha família, incluindo o Hugito, por todo o apoio e carinho.

Je remercie très spécialement Virginie, pour tout son amour, sa douceur et son soutien. Merci pour tout... Je remercie aussi toute la famille Glippa pour son amitié.

Agradeço muito especialmente às minhas super irmãs. Felizmente vocês existem e estão tão presentes. Sem a vossa alegria, dedicação e apoio, tudo teria sido muito mais difícil…

Quero finalmente agradecer à minha maravilhosa Mãe e ao meu querido Pai por todo o amor, apoio e pela total confiança que depositaram em mim durante todos este anos. Este trabalho é dedicado a vocês os dois…

Miguel

Abstract

Full understanding of the evolution and ecological role of an organism requires comprehension of its life cycle. This thesis focuses on the life cycle of the haptophytes, an ecologically important group of mainly marine microalgae. Present knowledge indicates that this group undergoes a haplo-diplontic sexual life cycle characterized by morphologically distinct haploid and diploid phases, each capable of independent asexual growth.

The first part of the thesis was dedicated to investigation of the interaction of the two life cycle phases of the ubiquitous coccolithophore *Emiliania huxleyi* (Calcihaptophycidae, Haptophyta) with specific viruses (*Eh*Vs) and comparison of the ecological dynamics of the two phases of this species in nature. The first study demonstrated that the non-calcified haploid phase of *E. huxleyi* is resistant to *Eh*Vs that infect and kill the calcified diploid phase and are responsible for termination of large-scale *in situ* blooms of diploid cells. These results help explain the evolutionary ecology of both host and virus, i.e. why *E. huxleyi* diploid cells can concentrate resources on rapid growth and *Eh*Vs remain highly virulent and also an ecological role of haploid cells (ensuring maintenance of the species when *Eh*Vs are abundant). In a wider context, such host-virus dynamics could account for the maintenance of the sexual life cycle and evolution of different morphologies within a life cycle. For field studies, a new method ("COD-FISH") was developed combining Fluorescent *In Situ* Hybridization (FISH) and cross-polarized microscopy, which allows detection of a given species at the genetic level and in parallel characterize morphologically (calcifying vs non-calcifying) the targeted cells. With a species level probe, COD-FISH allows quantification of *E. huxleyi* life cycle stages with different morphologies in seawater samples. COD-FISH analysis of samples from various marine settings revealed that diploid calcified cells were always numerically dominant, whereas the non-calcified phases (including haploid and diploid cells) were always present as background populations (ca. 5-40% of total cells) following the same dynamics as the diploid cells. In a series of preliminary laboratory culture experiments in which diploid and haploid cultures were mixed, diploid cells tended to out-compete and dominate haploid cells. Synthesis of these studies leads to the conclusion that in nature the diploid phase is probably highly competitive and dominates the haploid phase, the presence of the latter maybe being associated with a high life cycle turnover. This strategy would allow *E. huxleyi* to rapidly respond to environmental abiotic and biotic stresses, including massive viral infections.

In the second part of the thesis, two new life cycle associations in the coccolithophore family Pontosphaeraceae (Calcihaptophycidae, Haptophyta) are presented. These results augment our present knowledge of the diversity of coccolithophore life cycles and further reinforce the current hypothesis that the haplo-diplontic life cycle is ubiquitous among haptophytes. In the last study presented in this thesis, one culture strain of the Pavlovophyceae (Haptophyta) was demonstrated to be diploid. This is the first evidence relating to ploidy level in the Pavlovophyceae, which is a group that diverged early in the history of the Haptophyta. This study therefore provides new insights into the possible origins and early evolution of the haptophyte haplo-diplontic life cycle.

Among other perspectives, the final section highlights that development of the COD-FISH method opens an array of new technical possibilities to define more morphological associations and further explore the ecological role of life cycles in calcihaptophytes.

Key words

Life cycle, haptophytes, haplo-diploidy, physiology, ecology, diversity

Résumé

Une compréhension globale de l'évolution et du rôle écologique d'un organisme requiert la connaissance de son cycle de vie. Cette thèse se concentre sur le cycle de vie des haptophytes, un groupe écologiquement important de microalgues, principalement marines. Les connaissances actuelles indiquent que ce groupe effectue un cyle de vie sexuel haplo-diploïde caractérisé par des phases haploïde et diploïde morphologiquement différentes, chacune étant capable d'une croissance par reproduction asexuée produisant des populations indépendantes.

La première partie de cette thèse est dédiée à la compréhension de l'interaction des deux phases du cycle de vie du coccolithophore ubiquiste, *Emiliania huxleyi* (Calcihaptophycidae, Haptophyta) avec ses virus spécifiques (*Eh*V's) ainsi que la comparaison de la dynamique écologique des deux phases dans le millieu naturel. La première étude démontre que la phase haploïde non calcifiée de *E. huxleyi* est résistante aux *Eh*V's qui infectent et tuent la phase diploïde calcifiée. Ces virus sont également responsables, à grande échelle, de la disparition des *blooms* des cellules diploïdes. Ces résultats permettent de mieux comprendre l'écologie évolutive de l'hôte et du virus. De plus, ces résultats suggèrent que les interactions hôte-virus permettraient le maintien du cycle de vie sexué et l'évolution des différentes morphologies dans chacune des deux phases. Pour les études écologiques, une nouvelle méthode ("COD-FISH") a été développée, combinant l'hybridation i*n situ* fluorescente (FISH) et la microscopie polarisée. Cette méthode permet la détection d'une espèce avec une sonde moléculaire et en parallèle, de caractériser morphologiquement les cellules ciblées (calcifiantes ou non-calcifiantes). En utilisant une sonde spécifique pour *E. huxleyi*, sur des échantillons naturels, on a révélé que les cellules diploïdes calcifiantes sont toujours majoritaites, alors que les phases non calcifiantes (incluant les cellules haploïdes et diploïdes) sont toujours minoritaires (ca. 5-40% des cellules totales). De plus, les deux types de cellules suivent la même dynamique de croissance. Dans une série d'expérience *in vitro*, dans lesquelles les cellules haploïdes et diploïdes ont été mélangées, les cellules diploïdes se développent plus et prennent le dessus sur les cellules haploïdes. La synthèse de ces études permet de conclure que dans le millieu naturel, la phase diploïde est probablement plus compétitive et domine la phase haploïde. La présence des deux phases s'expliqueraient par une fréquence élevée du cycle de vie. Cette stratégie devrait permettre à *E. huxleyi* de répondre plus rapidement aux stress biotiques et abiotiques environnementaux, incluant les infections virales massives.

Dans la deuxième partie de cette thèse, on présente l'identification de deux associations entre les phases du cycle de vie dans la famille des Pontosphaeraceae (Calcihaptophycidae, Haptophyta). Ces résultats enrichissent nos connaissances sur la diversité des cycles de vie des coccolithophores et renforcent l'hypothèse actuelle que le cycle de vie haplo-diploïde est ubiquitaire au sein des haptophytes. Dans la dernière étude présentée dans ce travail de thèse, on a démontré qu'une souche de la classe des Pavlovophyceae était diploïde. Ces résultats constituent la première mise en évidence du niveau de ploïdie d'une Pavlovophyceae. Ce groupe ayant divergé très tôt dans l'histoire évolutive des haptophytes, ces résultats fournissent de nouvelles pistes sur les origines des cycles de vie haplo-diploides des haptophytes.

Dans la partie finale du manuscrit, on met en évidence le potentiel de la méthode COD-FISH pour étudier les cycles de vie des coccolithophores.

Mots Clés
Cycle de vie, haptophytes, haplo-diploïdie, physiologie, écologie, diversité

Resumo

Para uma compreensão global da evolução e função ecológica de um determinado organismo é importante ter em conta as diferentes fases do seu ciclo de vida. Esta tese é dedicada ao estudo das haptófitas, que é um grupo de microalgas de grande importância ecológica, que colonizam principalmente ambientes marinhos. Uma das suas características principais é possuírem um ciclo de vida sexual haplo-diplóide, onde as duas fases, a haplóide e a diplóide, são morfologicamente distintas e são capazes de se multiplicarem assexuadamente, produzindo populações independentes.

A primeira parte desta tese é dedicada ao estudo *in vitro* da interacção das duas fases do ciclo de vida do cocolitóforo *Emiliania huxleyi* (Calcihaptophycidae, Haptophyta) com vírus específicos (*Eh*V) e, posteriormente, ao estudo da dinâmica ecológica dessas mesmas duas fases no meio natural. No primeiro estudo, demonstrou-se que as células haplóides que desprovidas de cocólitos (não-calcificadas) são resistentes aos *Eh*Vs, enquanto que as células diplóides que possuem cocólitos (calcificada) são infectadas e mortas pelos *Eh*Vs, que já no meio natural são os agentes responsáveis pelo controlo e termino de *blooms* (afloração exponencial) produzidos por populações diplóides. Estes resultados aprofundam o conhecimento sobre as interacções ecológicas entre o hospedeiro *E. huxleyi* e o vírus *Eh*V e, num contexto mais vasto, sugerem que este tipo de interacções bióticas podem estar na origem da manutenção dum ciclo sexual e no consequente desenvolvimento de duas morfologias distintas em cada umas das fases do ciclo de vida da *E. huxleyi*. Para o segundo estudo realizado com amostras do meio natural, desenvolveu-se um novo método ("COD-FISH") que combina a Hibridação *In Situ* por Fluorescência (FISH) e a microscopia óptica de polarização cruzada. Este método, permite detectar uma dada espécie com uma sonda molecular e em paralelo caracterizar morfologicamente (calcificada *vs* não calcificada) as células detectadas. Deste modo, revelou-se que a fase diplóide (calcificada) é sempre dominante, enquanto que a fase não calcificada (que no caso de *E. huxleyi* pode ser haplóide ou diplóide) se apresentava sempre em menor número (ca. 5-40% da população total de *E. huxleyi*). No entanto, ambas as fases desenvolviam-se em paralelo, não sendo detectada uma sazonalidade no crescimento de cada uma delas. Noutro estudo, efectuado no laboratório verificou-se que quando as duas fases (diplóide e haplóide) eram misturadas na mesma cultura, a fase diploide sobrepunha-se à fase haplóide, dominando em número a cultura. O conjunto destes trabalhos, levaram-nos a concluir que nos oceanos a fase diplóide é muito competitiva e domina as populações de *E. huxleyi*. Por outro lado, a presença constante duas fases, poderá dever-se a uma elevada dinâmica do ciclo sexual. Esta estratégia permitiria às populações de *E. huxleyi*, de responder mais rapidamente a pressões ambientais, abióticas e bióticas, incluindo infecções virais.

A segunda parte desta tese de doutoramento começa pela descrição das duas primeiras associações de ciclo de vida da família Pontosphaeraceae (Calcihaptophycidae, Haptophyta). Estes resultados enriquecem o conhecimento actual sobre a diversidade dos ciclos de vida dos cocolitóforos, reforçando a hipótese de que o ciclo de vida do tipo haplo-diplóide é característico de todos os elementos deste grupo de organismos. No último estudo efectuado durante este trabalho de doutoramento, demonstrou-se que uma estirpe da classe Pavlovophyceae é diplóide. Este resultado, constitui a primeira evidência do nível de plóidia de uma microalga Pavlovophyceae, que é uma classe mal conhecida e que divergiu do resto das haptófitas muito cedo na história evolutiva desta Divisão. Este estudo fornece portanto, novas pistas sobre as origens do ciclo de vida haplo-diplóide das haptófitas.

Na parte final do manuscrito são debatidas as aplicações potenciais do método COD-FISH no estudo dos ciclos de vida dos cocolitóforos.

Palavras chave

Ciclo de vida, haptophytas, haplo-diplóidia, fisiologia, ecologia, diversidade

Table of Contents

PART III. OTHER STUDIES ON HAPTOPHYTES LIFE CYCLES DIVERSITY AND EVOLUTION

ANNEXES

Abbreviations List

AC	Algobank culture collection (Université de Caen)
CCMP	Culture collection of marine phytoplankton (Bigelow, USA)
Chl	Chlorophyll
COD-FISH	$CaCO_3$ optical detection coupled with FISH
CPM or XPM	Cross-polarized microscopy
CTD	Oceanographic machine used to measure *in situ* the conductivity, temperature and density of the sea-water
DAPI	4',6'- Diamidino-2-phenylindole
DNA	Desoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
***Eh*V**	*Emiliania huxleyi* Virus
FISH	Fluorescent in situ hybridization
HET	Heterococcolithophore
HOL	Holococcolithophore
HRP	Horse radish peroxidase
iDAPI	Intensity of DAPI fluorescence
LSU or 28S	Large subunit of the ribosomal gene
MOI	Multiplicity of infection
My or Ma	Millions of years
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
RNA	Ribonuclei acid
RCC	Roscoff culture collection
SDS	Sodium dodecyl sulfate
SSU or 18S	Small subunit of the ribosomal gene
TNT	Tris, NaCl, Tween buffer
TSA	Tyramide signal amplification
UV	Ultra-Violet
2N	Diploid
N	Haploid
19' Hex	19' hexanoyloxyfucoxanthin

Figures and Tables List

PART I. *INTRODUCTION AND OBJECTIVES*

1. Preamble

The life cycle of a species is the succession of organisms connected by reproductive processes that repeats itself more or less regularly (Kondrashov, 1997). This broad definition can be generally observed in nature. All biological forms, i.e. eukaryotes, prokaryotes and viruses, possess a life cycle during which they appear from parental entities, multiply and reproduce, in some cases mixing their genetic information with a sexual partner and transmitting it to progeny, and finally die. A life cycle is therefore a dynamic spiral through which species evolve and diversify, rather than a non-mutable circle continuously returning to the initial stage. This whole pathway characterises all species and defines their role in the ecosystem, through the interaction of each life phase with the abiotic and biotic environmental surroundings, and eventually leads to species evolution and diversification. In these terms, only the full comprehension of an organism's life cycle can unveil its real significance in evolutionary, ecological and biogeochemical perspectives. It is within this overall context that this thesis will be defined.

This work will be dedicated primarily to the study of the haplo-diplontic sexual life history of Emiliania huxleyi (Haptophyta), including the two main (diploid and haploid) phases. The coccolithophore E. huxleyi is one of the most abundant and widespread eukaryotic microalgal species in modern oceans, contributing significantly to marine food-web structure, and is currently considered as one of the key players in the global carbon and sulphur biogeochemical cycles, which influence global climate. Nevertheless, presently there is very little information available concerning the biology of its life cycle. During this thesis I will direct my research mainly in two complementary directions: firstly, exploration of the role of the life cycle in the ecology of this species in the natural environment; and secondly, comparing the physiological properties of life cycle phases.

During the course of this thesis, research concerning the life cycles of other haptophytes will be also performed. These complementary studies, some clearly exploratory, widened the scope of my thesis, facilitating general considerations on the evolution, diversity and ecology of haptophyte life strategies.

Therefore, in the following introductory chapter, general aspects of the eukaryotic life cycle will be exposed. Then, the state-of-the-art of knowledge of haptophyte life cycles will be reviewed, with special emphasis on the life cycle of E. huxleyi. The chapter will be concluded with a statement of the objectives of the thesis and a summary of the content of the manuscript.

2. Introduction

2.1. Eukaryotic life cycles: general overview

2.1.1. Diversity

The recognized sexual life cycle involves the fusion (sex or syngamy) of two cells each possessing one set of chromosomes (haploid cells) leading to the production of a new cell with two sets of chromosomes (diploid cell). This cell will then divide by meiosis, during which the new cell recombines the two parental genomes (crossing-over) and divides into four daughter cells potentially possessing genes from both initial entities. The key function of this sexual process among living organisms is classically thought to be associated with the creation of genetic variation, which allows species to produce new phenotypic diversity in offspring and hence have a better capacity to adapt to a changing environment or purge the genome form deleterious mutations or even repair DNA damages through recombination (e.g.(Maynard Smith & Szathmáry 1999)). However, sexual reproduction implies several considerable 'costs' for the species that undergo this process. For examples, asexual females can produce twice as many genetically identical daugthers compared to sexual females who must allocate half of their reproductive potential to male offspring, sexual species need to find a partner with which to fuse and perform meiosis which is energetically costly. Therefore a wide range of alternative theories have been posited throughout the last century to try to explain the evolutionary and ecological advantages and disadvantages of sex, making this issue one of the most enduring puzzles in evolutionary biology (Bell 1982, Hurst & Peck 1996, Maynard Smith & Szathmáry 1999, Cavalier-Smith 2002, Otto & Lenormand 2002, Hoekstra 2005). The fact is that a large majority of eukaryotic species undergo this sexual cycle with a consequent alternation of haploid and diploid generations.

The wide variety of sexual life cycles found in nature share this basic structure, but exhibit differences in two main parameters: the temporal importance of each phase (i.e. the proportion of time spent in the haploid or the diploid phase), and the degree of mitotic activity in each phase (i.e. asexual multiplication of haploid and/or diploid cells) (Bell 1982, Mable & Otto 1998). Three basic types of sexual life cycle are found in eukaryotes (Fig. 1): The diplontic (or diploid), the haplontic (or haploid) and the haplo-diplontic (or haplo-diploid) life cycle. These cycles differ with regard to the relative position of meiosis and syngamy. In the diplontic life cycle, syngamy directly follows meiosis, the multiplication and growth (somatic development in multicellular organisms) occurs only in the diploid generation and the gametes are the only haploid cells. Conversely, in the haplontic life cycle, meiosis directly

follows syngamy and multiplication occurs in the haploid phase, with a minimal diploid phase corresponding solely to the zygote. Finally, when meiosis and syngamy are separated in time, development can occur both in the haploid and diploid phases, and the cycle is termed haplo-diplontic. In such biphasic life cycles, the two phases can either be dependent (with one phase growing on the other) or independent, and they can vary from extreme heteromorphy (large morphological differences) to isomorphy (morphologically identical) (Bell 1982, Mable & Otto 1998) (de Reviers 2002).

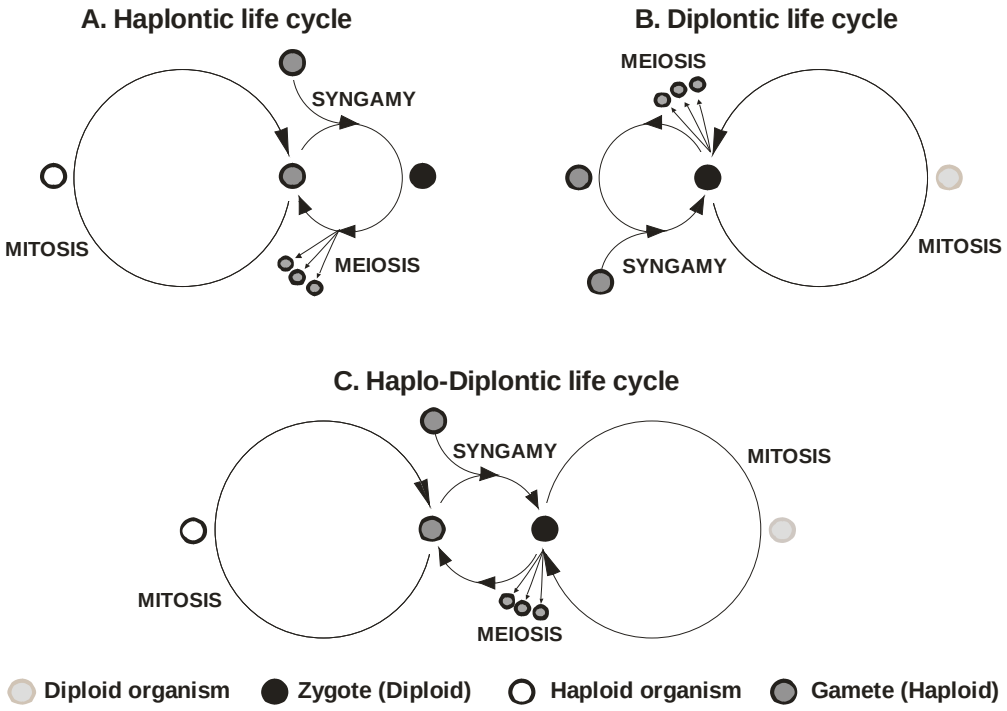


Figure 1. Theoretical representations of the three basic types of life cycle strategies present in eukaryotes (based on (Mable & Otto 1998)).

When multiple characters (i.e. degree of dependence, morphological differentiation between phases, etc.) are taken into account, the range of eukaryotic life cycles is enormous (Bell 1982, Van Den Hoek et al. 1995, Kondrashov 1997, Mable & Otto 1998, de Reviers 2002, Coelho et al. 2007). Describing this diversity is beyond the scope of this thesis. It is important to note, however, that within the rigid framework defined above, and accounting for the present very incomplete state of knowledge, the phylogenetic distribution of the three core life cycles appears to be very wide (Fig. 2). A generic conclusion that can be drawn from this synthesis is that it is difficult to define an origin (even though some hypotheses state that the ancestral state is probably the haplo-diplontic life cycle, (Maynard Smith & Szathmáry 1999)) and major evolutionary trends in transition between life cycle types. In general, multicellular organisms tend to have diplontic life cycles (or at least dominant diploid phases like higher

plants), whilst 'simpler' organisms present all sorts of life cycle types. (Bell 1982), looking closer within each group, proposed that when there is a clear tendency in the potential for growth of either haploids or diploids within a phylum (or other high level taxonomic group), this trend is characteristic of all of its members (e.g. all charophytes are haplontic, all chordates are diplontic, all diatoms are diplontic). In contrast, in groups where species can grow as both diploids and haploids, life cycles may vary (e.g. chlorophytes, phaeophytes, fungi). This suggests that life cycle strategies can be a selectively variable trait, capable of continued evolutionary modifications, but once one phase is dominant, it may be difficult to expand the alternative ploidy level.

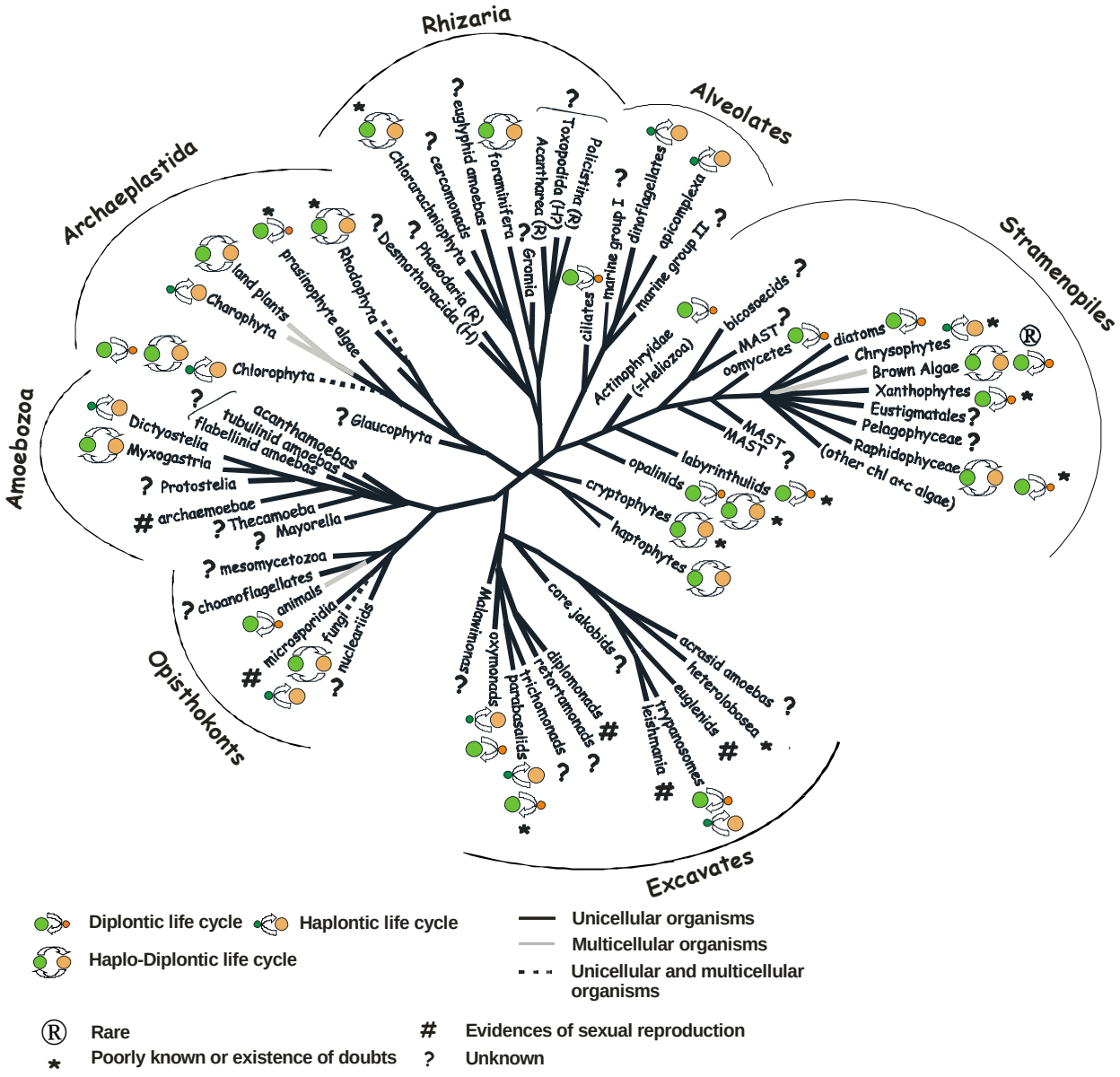


Figure 2. Diversity of life cycles strategies in the eukaryotes (the tree topology was based on: (Baldauf 2003, Burki et al. 2007, Hackett et al. 2007)); Information about the diversity of life cycles was retrieved from: (Bell 1982, Van Den Hoek et al. 1995, Kondrashov 1997, Mable & Otto 1998, de Reviers 2003, Nuismer & Otto 2004).

Closer attention should be paid in the future to the wide diversity of unicellular taxa for which new information (including genetic and genomic data) are now available. This approach would improve the present understanding of the evolution and maintenance of life cycle traits in the eukaryotes

2.1.2. Theoretical assumptions

For decades, the fact that almost all metazoans and higher plants undergo their development in the diploid phase strongly skewed scientific perception towards the supposed ecological and evolutionary advantages of diploidy (Valero et al. 1992). However, the other two life cycle strategies persist and are widely spread throughout the diversity of organisms (Fig. 2). As a result, attention has turned towards understanding the evolution, maintenance and persistence of the diversity of life strategies. This shift has led to numerous theoretical predictions about the advantages and/or disadvantages of haplonty, diplonty and haplo-diplonty:

Advantages of diplontic life cycles

One of the most striking genetic consequences of being diploid is that nearly all deleterious mutations (if recessive) within the genome are masked by a dominant functional allele. By contrast, every mutation within haploid genomes is expressed and immediately subjected to selection (which may or may not be disadvantageous, see below). Consequently, individuals with a prolonged diploid phase should have higher fitness and diploidy should be favoured (Perrot 1994).

Having two copies of each gene may also speed-up the rate of adaptation of diploid organisms to new environments. Not only do diploids harbour twice as many genes capable of beneficial mutations, but since diploids mask deleterious mutations, they tend to have larger genetic pools, which can be a source of variability (e.g. new functions) that may be beneficial and selected to meet a changing environment (Orr & Otto 1994, Mable & Otto 1998).

Recently, Nuismer and Otto (Nuismer & Otto 2004), suggested also that host-parasite interactions may favour diploidy in the host, allowing them to harbour a greater diversity of recognition molecules that may prevent infections. The corollary of this is that haploidy would be favoured in parasites in order to express as few recognition molecules as possible and thus avoid recognition and suppression by hosts.

Advantages of haplontic life cycles

Some of the advantages of diploidy can under certain circumstances be disadvantageous. Masking, for instance, can be disadvantageous to the offspring of a diploid organism which inherits deleterious mutations. Conversely, deleterious mutations are more efficiently eliminated in haploids, and therefore a longer haploid phase can be favoured. However, theoretical models predict that if there is a high degree of genetic mixing within a haploid population, the advantages of haploidy (few mutations) will be shared with some existing diploid members of the population, who then gain advantages both of fewer deleterious mutations and of masking (Mable & Otto 1998).

Lewis (Lewis 1985) proposed that haploid cells may have a nutritional advantage over diploid cells. Haploid cells are typically smaller than diploid cells and thus have a higher surface area to volume ratio and since the ability to transport nutrients across the cell membrane depends on surface area, this increased ratio may lead to improved growth and/or survival in nutrient-poor conditions. In addition, haploid cells, since they have half the DNA content of diploids, would have lower energetic costs for division and growth (Lewis 1985), (Mable & Otto 1998)). Cavalier-Smith (Cavalier-Smith 1978) presented a similar type of hypothesis, suggesting that the evolution of ploidy levels may be a by-product of selection for optimal nuclear or cell size. Conditions favouring smaller individuals would select for haploid life cycles, whereas conditions favouring larger individuals would favour diploid cycles.

Advantages of haplo-diplontic life cycles

The hypotheses reviewed above favour haploidy under some circumstances and diploidy under others. It could be expected that these selective forces could occasionally balance, favouring life cycles with both an extended haploid and diploid phase. Theoretical models, however, do not predict this scenario (Mable & Otto 1998).

One potential advantage of haplo-diplonty is an increased scope for utilization of resources. For instance, in the presence of disruptive selection (biotic or abiotic) favouring two distinct phenotypes, a species may evolve a heteromorphic life cycle (or two phases with different physiological properties that may not be immediately visible, but lead to a different ecological ‘behaviour’) with the haploid and diploid phases occupying different ecological niches (Destombe et al. 1992, Valero et al. 1992, Mable & Otto 1998, Hughes & Otto 1999, Thornber 2006). A similar argument posits that haplo-diplonty allows for two modes of dispersal of the genetic material (associated with different survival and/or dispersal properties of life cycle phases) (Mable & Otto 1998).

This brief and non-exhaustive review demonstrates that there are a considerable amount of hypotheses that have been generated to attempt to explain the wide range of sexual life cycles found in nature. Strikingly, however, there are very few reports of experimental studies aimed at testing these hypotheses, and those which exist have concentrated on a few groups of organisms, notably metazoans (Mable & Otto 1998, Coelho et al. 2007). This is probably linked to the poor state of knowledge of the basic biology of many organisms and to limitations of growing them in culture, which is often a prerequisite for performing accurate experimentation. Nevertheless, theoretical modelling provides a useful framework that may help interpretation of observations made in nature.

2.2. The Haptophytes

The Haptophyta is a monophyletic division that includes a wide diversity of photosynthetic organisms possessing chlorophyll *a* and *c* as the main photosynthetic pigments (Jeffrey & Wright 1994) (with the exception of a few Artic species that appear to lack chloroplasts, (Marchant & Thompson 1994)). According to recent molecular 'clock' studies, this group appeared more than 1000 My ago (Yoon et al. 2004, de Vargas et al. 2007), branching very deep in the eukaryotic tree of life, close together with the cryptophytes (e.g.(Burki et al. 2007, Hackett et al. 2007) (see Fig. 2), at the base of the stramenopiles+ alveolates+ rhizaria ('SAR') mega-clade. Presently, the haptophytes are divided into two classes: the more diverse Prymnesiophyceae (6 orders) and the Pavlovophyceae (1 order) (Cavalier-Smith 1994, Jordan & Kleijne 1994, Edvardsen et al. 2000, de Vargas et al. 2007) (Table 1).

Table 1. Haptophyta Classes and Orders (Jordan et al. 2004)

Class Pavlovophyceae (Cavalier-Smith, 1986) Green and Medlin in (Edvardsen et al. 2000)
Order Pavlovales Green, 1976
Class Prymnesiophyceae Hibberd, 1976; emend. Cavalier-Smith in (Cavalier-Smith et al. 1996)
Order Phaeocystales Medlin in Edvardsen et al., 2000
Order Prymnesiales Papenfuss, 1955; emend. Edvardsen and Eikrem in Edvardsen et al., 2000
*** Order Coccolithales** Schwarz, 1932; emend. Edvardsen and Eikrem in Edvardsen et al., 2000
*** Order Syracosphaerales** Hay, 1977; emend. (Young & Henriksen 2003)
*** Order Zygodiscales** (Young & Bown 1997)
*** Order Isochrysidales** Pascher, 1910; emend. Edvardsen and Eikrem in Edvardsen et al., 2000
* ***incertae sedis*** taxa will be considered together with the coccolithophore orders

*Calcified Prymnesiophyceae, the coccolithophores (subclass Calcihaptophycidae, (de Vargas et al. 2007)

The organisms in this division are generally distinguished, among other characters (see Fig. 3), by the presence of an unique organelle called the haptonema (from the Greek *hapsis*-touch), which is superficially similar to a flagellum, but differs in the arrangement of its microtubules and is used for prey capture, or attachment to substrates, or even as a sensory appendix (Inouye & Kawachi 1994).

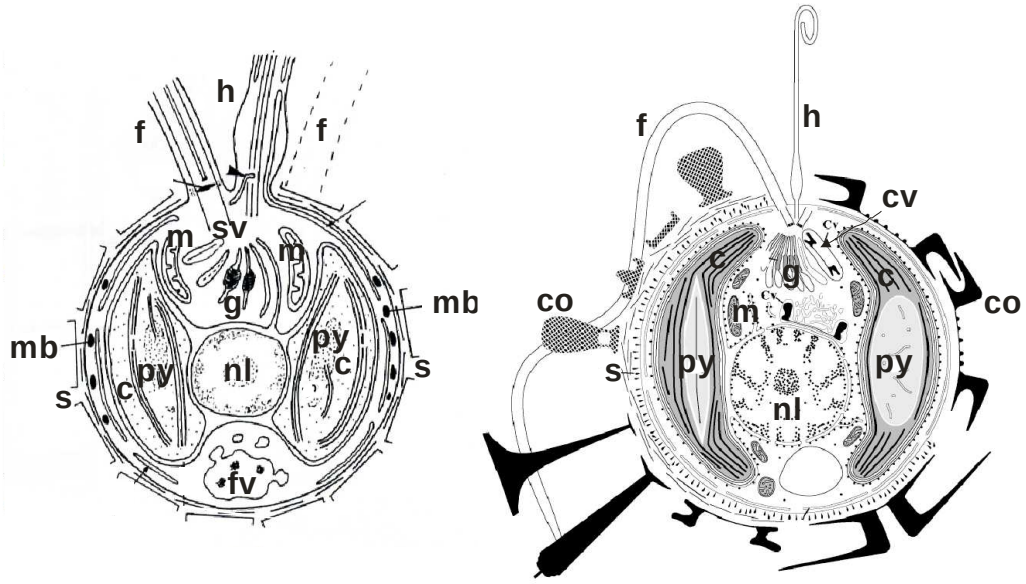


Figure 3. Schematic representation of a section through a typical non-calcified prymnesiophycean cell (left, modified from (Jordan et al. 1995)) and a Calcihaptophycidae (right, modified from (Billard & Inouye 2004) showing the principal organelles. c, chloroplasts; co, coccoliths; f, flagella; h, haptonema; fv, vacuole containing ingested material; g, Golgi body with dilated cisternae ('peculiar Golgi') and nascent scales (scale vacuole, sv) or coccolith (coccolith vacuole, cv) (produced intracellularly); mb, muciferous bodies; nl, nucleus; py, pyrenoid; s, body-scales.

In terms of diversity, there are presently more than 350 described haptophyte species (Jordan et al. 2004) that occur principally as solitary unicellular free-living cells, but may form colonies of motile cells, pseudo-filaments or mucous-bound aggregations of non-motile cells, and may even live in symbiotic associations with foraminifera and acantharians (Jordan & Chamberlain 1997, Gast et al. 2000, Lee 2006).

The haptophytes have a fairly ubiquitous distribution (Thomsen et al. 1994, Winter et al. 1994, Jordan & Chamberlain 1997, Not et al. 2005), being known principally from marine environments, with a few freshwater and terrestrial representatives (Green et al. 1996, Edvardsen et al. 2000, de Vargas et al. 2007). Some species form large blooms, highlighting the haptophytes as important primary producers. Moreover, some species, the 'coccolithophores' (or Calcihaptophycidae, (de Vargas et al. 2007)), produce calcified scales (coccoliths), which, upon death of the cell, are liberated and may be transported to the sea floor where they constitute an important component of sediments (Steinmetz 1994). These calcareous deposits appeared in the Triassic and have constituted the major constituent of chalk since the late Cretaceous (65-95 My) , producing a well-defined fossil record with numerous applications in applied and fundamental stratigraphic and palaeoceanographic studies (Roth 1994, Bown & Young 1998). In the last thirty years, the extant coccolithophores

have gained special attention mainly due to the formation of blooms, whose reflectance via birefringent loose coccoliths can be detected by satellite imagery (Holligan et al. 1983, Balch et al. 1991). The processes of photosynthesis, calcification and respiration occuring in such large scale blooms have important implications for the global carbon cycle (Rost & Riebesell 2004) (Fig. 4). In addition, blooms of haptophytes (including non-coccolithophores such as *Phaeocystis*) play an important role in the global sulphur cycle through the production of dimethyl sulphide, which, once volatilized to the atmosphere, appears to contribute directly to cloud formation (Malin et al. 1994, Malin & Steinke 2004) (Fig. 4). In these terms, haptophytes make a large contribution to global climate regulation (Westbroek et al. 1993). Finally, some bloom-forming species can also produce toxins, mucilage or foam, causing locally severe environmental and economic problems (Moestrup 1994, Edvardsen 2001).

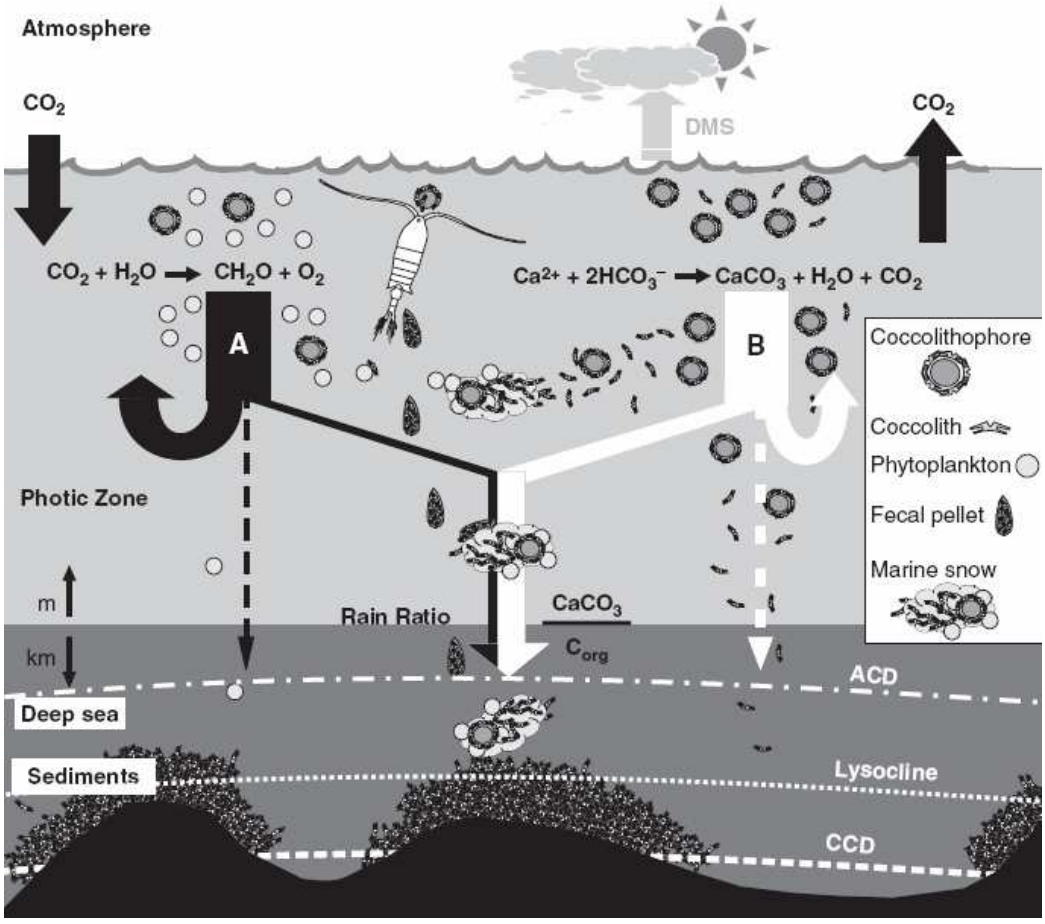


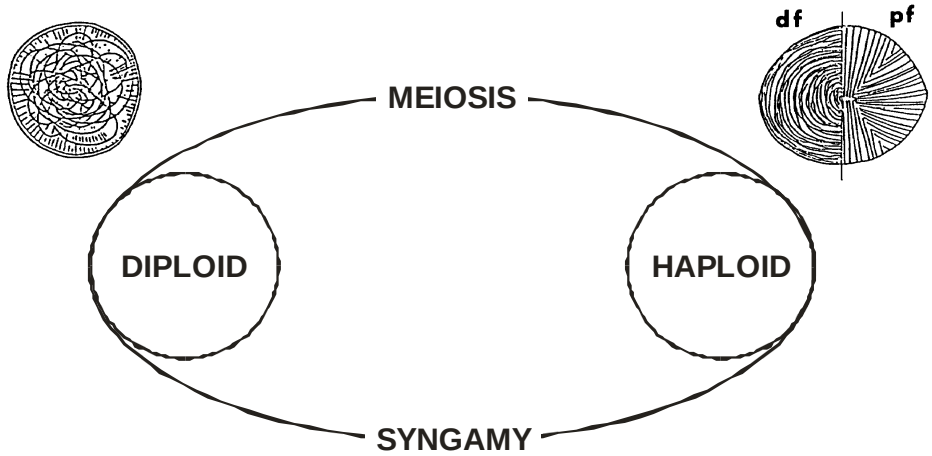
Figure 4. Schematic representation of the role of haptophytes in biogeochemical cycles of carbon and sulphur (de Vargas et al. 2007). Haptophytes participate actively in gas exchange (CO_2, DMS) between seawater and the atmosphere and to the export of organic matter and carbonate to deep oceanic layers and sediments. Coccolithophores, in particular, are the main actors of the carbonate counter-pump (B), which through the calcification reaction is a short-term source of CO_2. Via the ballasting effect of their coccoliths on marine snow, they are also the main drivers of the organic carbon pump (A), which removes CO_2 from the atmosphere. Ultimately, certain types of coccoliths

resistant to dissolution in the lower oceanic layers are deposited at the sea floor (fossil archive) (Rost & Riebesell 2004).

2.2.1 The life cycles of Haptophytes

Several heteromorphic life histories have been documented throughout the class Prymnesiophyceae in the past 50 years, mainly through the work of researchers including M. Parke, I. Manton, P. Gayral, J. Fresnel, C. Billard, J. Green, D. Klaveness, B. Edvardsen, A. Larsen, L. Cros, J. Young, I. Probert and A. Houdan, among others, and in the framework of multidisciplinary research consortia such as the CODENET and BOOM[1] that focussed on the basic biology, ecology, evolution and biogeochemical impact of haptophytes and especially coccolithophores. The majority of reported life histories include alternation between non-motile and flagellated stages or between two flagellated stages, and between colonial and single cell stages and between benthic and planktonic stages.

Reviewing the available data, Billard (Billard 1994) suggested that life cycles in the Prymnesiophyceae probably all included two morphologically dissimilar phases, one haploid and the other diploid, each capable of independent asexual reproduction, and that at some point would undergo sexual reproduction (fusion and/or meiosis) to form the complementary phase. This pattern characterizes a typical eukaryotic haplo-diplontic life cycle. This hypothesis was based on extrapolation from the fact that the different phases of coccolithophore taxa for which ploidy level had been determined (e.g. *Pleurochrysis carterae*, *Hymenomonas coronata*, *Coccolithus pelagicus*) had characteristic patterns of ornamentation of their organic (body) scales (Fig. 5).



[1] *CODENET*- Coccolithophorid Evolutionary Biodiversity and Ecological Network coordinated by Jeremy Young (Departement of Palaeontology, The Natural History Museum, London)
BOOM- Biodiversity of Open Ocean Microcalcifiers, coordinated by Colomban de Vargas (Centre National de la Recherche Scientific, Station Biologic de Roscoff)

Figure 5. Coccolithophore life cycle model based on scale ornamentation (Billard 1994). The body scales of the diploid heterococcolith-bearing cells have identical ornamentation on both sides, whereas those of the haploid stage have distinct patterns on the proximal (**pf**) and distal (**df**) (see also Fig.3).

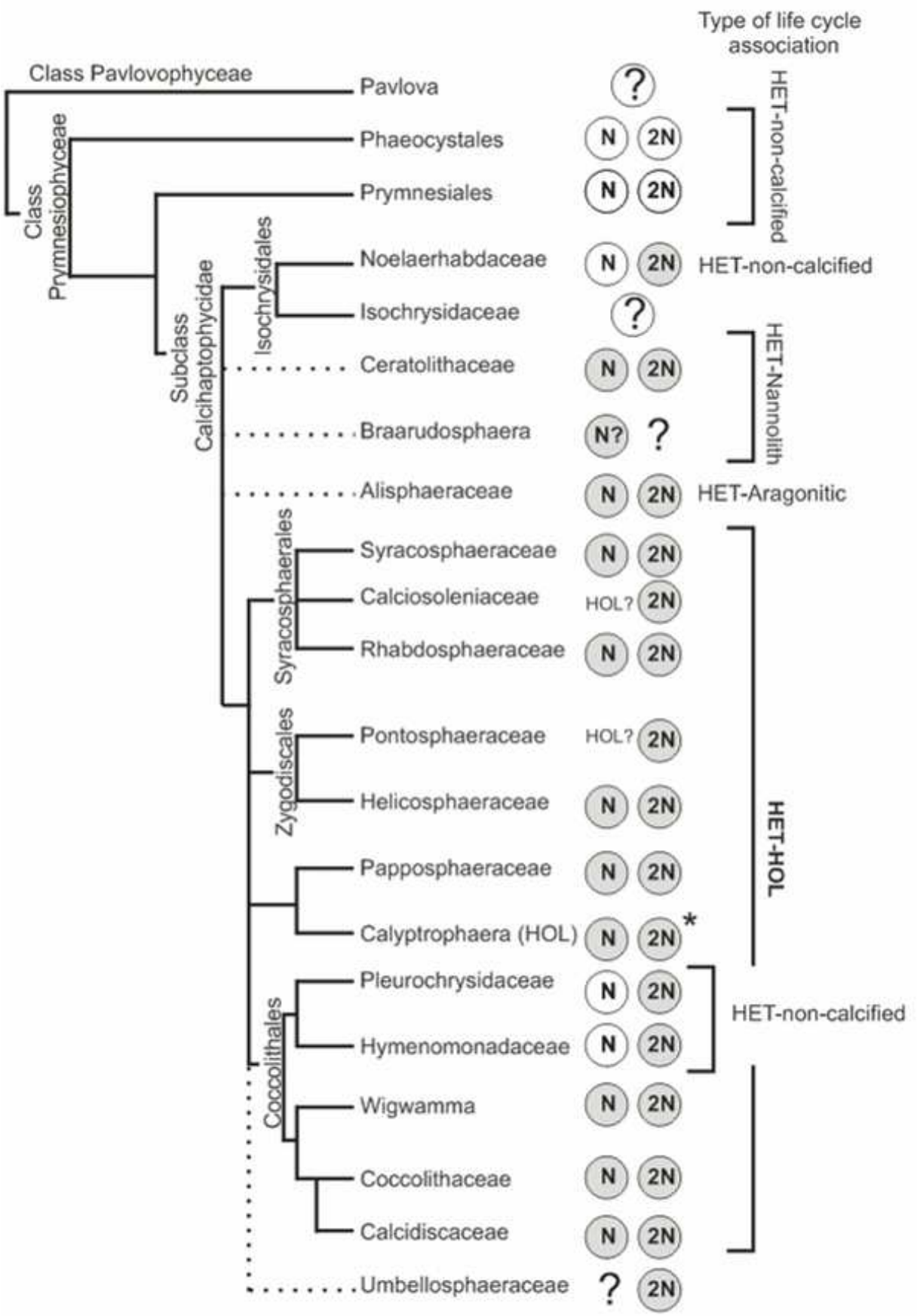
Subsequent observations have been reported from both cultured strains where sexuality was directly observed (Houdan et al. 2004, Noel et al. 2004) or the comparative ploidy level estimated (e.g. (Green et al. 1996, Houdan et al. 2004), and from field samples where life cycle transition stages ('combination coccospheres', see below) were found (e.g. (Cros et al. 2000)). With some possible exceptions (e.g. *Prymnesium parvum*), the majority of species analysed precisely fit the Billard model (Houdan et al. 2004). However, present knowledge of the functioning of these life cycles, including recognition of the triggers that induce phase changes (syngamy or meiosis), is extremely limited. Sexuality was induced in one case through dramatic physical and chemical manipulation of culture media (Noel et al. 2004), which was probably far from representing the real triggers acting in nature.

In summary, the current consensus of opinion is that all haptophytes exhibit a haplo-diplontic life cycle, but in fact information is still limited, particularly for non-calcified taxa (Fig. 6). Following the structure presented in Billard (Billard 1994), the state of knowledge on life cycles in the principal haptophyte groups will be reviewed in the following sections. While presenting this overview, questions that are particularly relevant in the frame of this thesis will be highlighted.

A. The Pavlovophyceae

Alternation of generations has, as yet, not been directly or indirectly demonstrated in any members of the Pavlovophyceae. One of the reasons for this is because the organisms within this class do not possess the ornamented plate scales that Billard (Billard 1994) proposed as an indicator of ploidy state in the Prymnesiophyceae. The Pavlovophyceae do possess simple organic 'knob scales' on the cell body and/or flagella and these do vary in morphology between described taxa, but this has never been correlated to ploidy level differences. Billard and Inouye (Billard & Inouye 2004) noted that some Pavlovophyceae are predominately non-motile, whereas others are predominately flagellated, and suggested that this may indicate different life cycle phases, but it is still not known whether a sexual life cycle occurs, and if so, whether it is haplo-diplontic like other haptophytes. It should be noted that it is entirely possible that the two possible stages do not differ morphologically (isomorphy). Characterisation of life cycle strategies in the Pavlovophyceae is important in the context of unveiling the origin of the haplo-diplontic life cycle strategy in the Haptophyta.

Did this life cycle strategy evolve in the Prymnesiophyceae, or is it a pleisiomorphy, having been present in the ancestral haptophyte prior to the divergence of the two known classes?


Type of life cycle association
Class Pavlovophyceae
Pavlova
Class Prymnesiophyceae
Phaeocystales
Prymnesiales
HET-non-calcified
Subclass Calcihaptophycidae
Isochrysidales
Noelaerhabdaceae
HET-non-calcified
Isochrysidaceae
Ceratolithaceae
HET-Nannolith
Braarudosphaera
Alisphaeraceae
HET-Aragonitic
Syracosphaerales
Syracosphaeraceae
Calciosoleniaceae
Rhabdosphaeraceae
Zygodiscales
Pontosphaeraceae
Helicosphaeraceae
Papposphaeraceae
Calyptrophaera (HOL)
HET-HOL
Coccolithales
Pleurochrysidaceae
Hymenomonadaceae
HET-non-calcified
Wigwamma
Coccolithaceae
Calcidiscaceae
Umbellosphaeraceae
N
2N
N?
HOL?
?
*

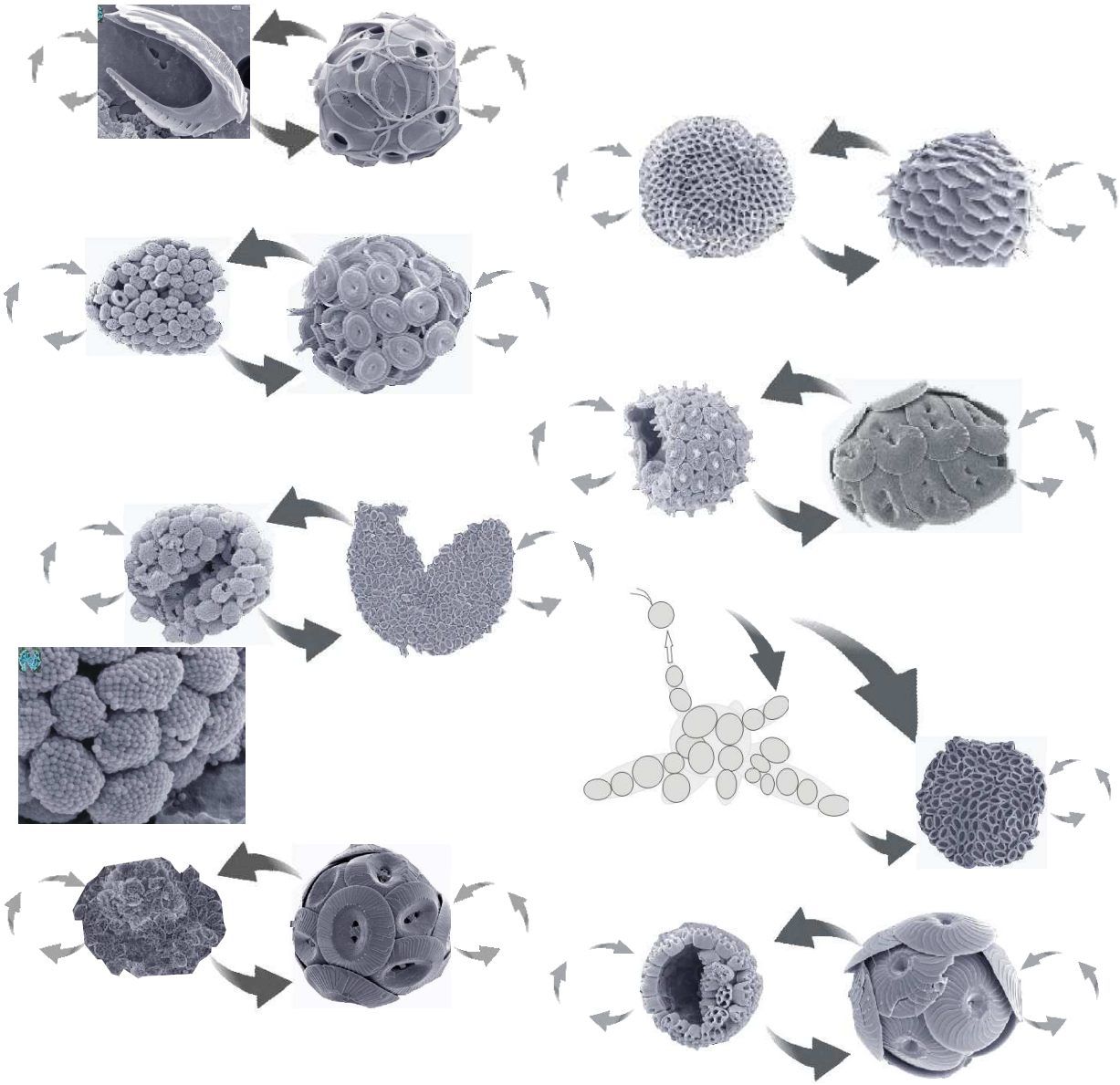


Figure 6. (*Previous page*) Schematic consensus tree of the relationship of extant Haptophyta families, based on molecular genetic data (based on (Houdan et al. 2004, Young et al. 2005, Takano et al. 2006, de Vargas et al. 2007)). The dotted lines represent groups for which the phylogenetic data is still unconclusive or is totally lacking. White circles represent non-calcified species or life cycle phases. Shaded circles indicate life cycle stages producing coccoliths (N – haploid phase, 2N- diploid phase). The symbol -?- represents cases for which the life cycle is unknown; HOL?, represents groups for which a holococcolithophore (haploid) life cycle phase is expected to exist, but has never observed. Although haplo-diplontic life cycles have been demonstrated from only a fraction of haptophytes, it seems likely from this phylogenetic distribution that this is the ancestral state across at least the class Prymnesiophyceae. The * represent the case of the Calyptosphaeraceae in which the diploid phase was only seen after culture manipulation (Noël et al. 2004).
(*This page*) Examples of life cycle associations in the Calcihaptophycidae (left- haploid; right- diploid). From top to bottom: *Ceratolithus cristatus vs Neosphaera coccolithomorpha*; *Alisphaera gaudii vs Polycrater gaudii*; *Syracosphaera pulchra* HOL (*Calyptrosphaera oblonga*) vs *S. pulchra* HET; *Helicosphaera carterae* HOL (*Syracolithus catilliferus*) *vs H. carteri* HET*; Calyptrosphaera sphaeroidea* HOL vs *C. sphaeroidea* HET; *Pleurochrysis carterae* vs *Apistonema* stage; *Coccolithus braarudii* HOL (*Crystallolithus braarudii*) vs C. braarudii; *Calcidiscus quadriperforatus* HOL (*Syracolithus quadriperforatus*) vs *C. quadriperforatus* HET (images retrieved from the PlanktonNet website: http://planktonnet.eu/).

The present availability of multiple Pavlovophyceae strains in culture (Algobank-Caen Culture Collection: http://www.unicaen.fr/algobank; Roscoff Culture Collection: http://www.sb-roscoff.fr/Phyto/RCC/) provides the experimental framework to explore this issue. During this thesis, the ploidy level of some Pavlovophyceae culture strains was studied and new findings are presented and discussed in Chapter 5.

B. The non-calcified Prymnesiophyceae

Alternations of generations with different ploidy levels are known in certain members of both clades of non-calcified Prymnesiophyceae (Table 1).

In the Phaeocystales species *Phaeocystis globosa*, Vaulot et al. (Vaulot et al. 1994) identified the presence of motile haploid flagellated cells, a non-motile diploid colonial stage and a diploid flagellated phase, but the dynamics of a sexual transition between these phases is still not fully understood. However, field observations suggested that the haploid phase is present in the water before the formation of blooms of diploid colony-forming cells. Sexual events (syngamy of haploid cells) could thus be involved in bloom initiation. The trigger has been hypothesized to be the availability of nutrients and/or light levels (Peperzak et al. 2000). During bloom development (vegetative growth) some diploid flagellated cells would be released from disrupted colonies due to turbulence, constituting the inoculum for new colonies. At the end of blooms, due to nutrient depletion or a rapid decrease in temperature, the colonies collapse and the cells are massively grazed and/or killed by infectious viruses (Baudoux & Brussaard 2005, Brussaard et al. 2005). During this termination process, new flagellated cells are released from the old colonies, probably including haploid cells, therefore completing the sexual cycle (Peperzak et al. 2000, Rousseau et al. 2007).

For the other five described *Phaeocystis* species information about life cycling is relatively scarce. However, different ploidy levels have been identified, suggesting the existence of a similar life strategy in all species (Jacobsen 2002, Rousseau et al. 2007).

In the Prymnesiales, *Chrysochromulina polylepsis* has been shown to have a haplo-diplontic life cycle (Edvardsen & Vaulot 1996) which involves the alternation between two flagellated forms, one haploid and the other diploid, that differ in body scale morphology and in cell size. Neither syngamy nor meiosis have been reported (Edvardsen & Vaulot 1996). The environmental factors that promote the transition between cell types remain unknown, but culture studies showed that the different life cycle phases have different temperature and irradiance preferences, and also differ in the ability to produce toxins, suggesting different

eco-physiological specializations and possibly the adaptation of each phase to different ecological niches (Edvardsen 2001).

Another species of Prymnesiales, *Prymnesium parvum*, has been shown to be the diploid phase in a life cycle, in which the haploid phase was originally described as a distinct species, *P. patelliferum* (Larsen & Edvardsen 1998). Both life cycle phases differ in minor details of scale morphology (Green et al. 1982), fitting the model proposed by Billard (1994) for the coccolithophores. The two forms often co-occur in nature and in the laboratory there are no apparent differences in response to different abiotic parameters (salinity, temperature and irradiance), which led to the conclusion that the two phases probably share the same niche (Larsen & Bryant 1998).

Recently, other haplo-diploid life cycle in a *Prymnesium* species presenting differences in scale morphology has been recorded, but there are no physiological or ecological information about these new life cycle associations (Probert & Fresnel 2007, Seoane et al. in press).

Finally, an enormous diversity of new ribosomal sequences (ribotypes) has recently been discovered within the '*Prymnesium-Chrysochromulina*' clade B2 (Annex 5), these being widely spread throughout the oceans. It should be thus interesting to unveil the role of sexuality for the evolution and maintenance of such wide diversity.

C. The Calcihaptophycidae

A relatively large amount of information on life cycles is available for the Calcihaptophycidae (or the coccolithophores), due mainly to the possession of visible and identifiable morphological characters, the coccoliths. Like the other haptophytes, the coccolithophores multiply and produce their populations generally by mitosis (vegetative multiplication). The arguments for the existence of a sexual haplo-diplontic life cycle are based on various direct and indirect evidence: observation of phase changes and sexuality (syngamy or meiosis) in culture (e.g. (Parke & Adams 1960, Gayral & Fresnel 1983, Houdan et al. 2004, Houdan et al. 2006)) examination of body scale ornamentation by transmission electronic microscopy (Billard 1994); nuclear staining and relative chromosome counts of cultured species (Fresnel 1989) or flow cytometric analysis of relative ploidy level (Green et al. 1996, Houdan et al. 2004); and finally the observation of numerous combination coccospheres in field samples (e.g. (Kleijne 1991, Thomsen et al. 1991, Cortes 2000, Cros et al. 2000, Geisen et al. 2002)), which are interpreted as capturing the instant of a life cycle phase transition (Fig. 7).

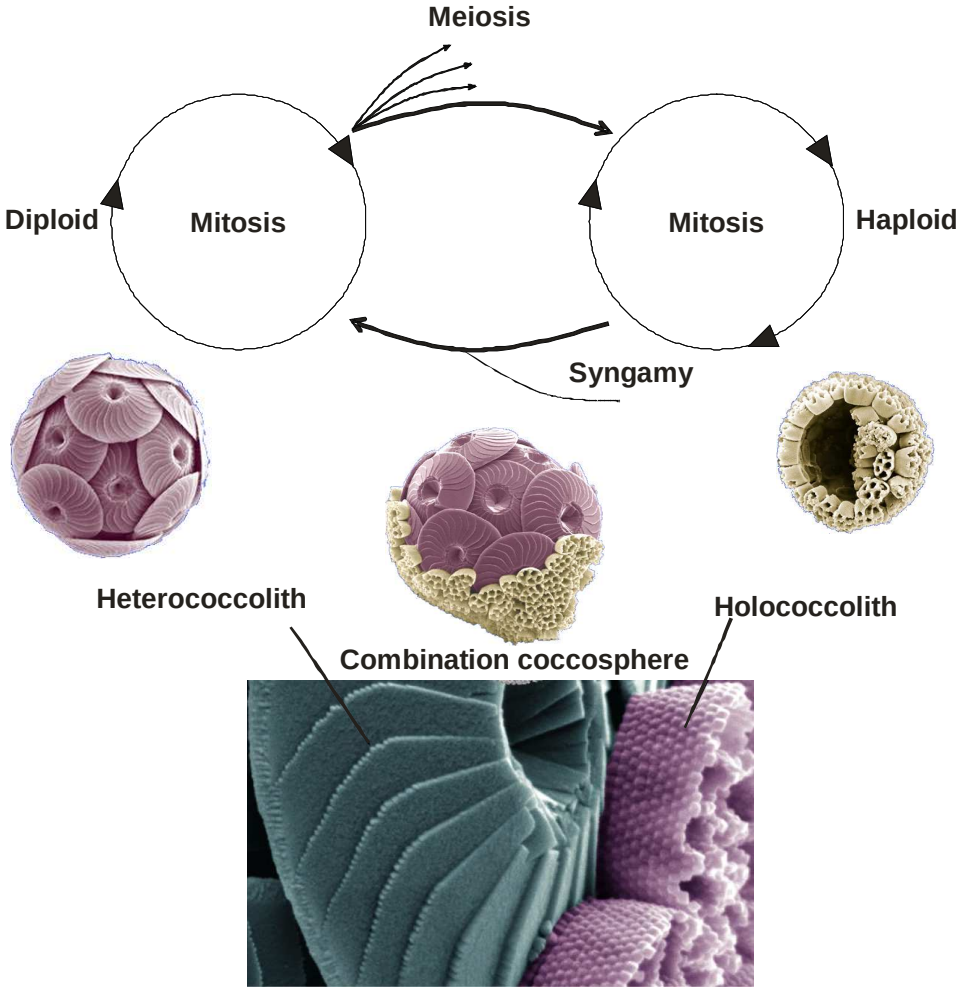


Figure 7. Schematic representation of a typical heteroccolithophore-holococcolithophore life cycle, using *Calcidiscus quadriperforatus* as example (based in (Young et al. 2005)). The dominant reproduction process is mitosis (asexual multiplication), which occurs in both the haploid and diploid phases. Transitions between the phases through meiosis or syngamy occur only rarely and the triggers that induce these transitions are unknown. However, occasionally transition phases, referred to as combination coccospheres, are observed in natural water samples. The illustrated combination coccosphere has a heterococcolith-bearing, and so diploid, cell emerging from inside a covering of holococcoliths inherited from fusion (syngamy) of two haploid cells. The morphological differences and the details of the hetero- and holococcoliths are illustrated at the bottom of the figure.

On current evidence it is possible to define four main types of hetermorphic digenetic life cycle associations in the Calcihaptophycidae (see also Fig. 6):

Heterococcolithophore-holococcolithophore

This type of life cycle association is common for the majority of the oceanic coccolithophore families (Fig. 6, Helicosphaeraceae, Syracosphaeraceae, Rhabdosphaeraceae, Coccolithaceae, Calcidiscaceae and Pappposphaeraceae). It involves the association two phases

characterized by different modes of calcification, i.e. the heterococcolithophore phase in which the coccoliths (heterococcoliths) are produced intracellularly (Brownlee et al. 1994) and consist of discs or rings constructed from one or more radial arrays (cycles) of elaborate and variable-shaped crystal units (Young et al. 1992), and the holococcolithophore phase, in which the coccoliths (holococcoliths) are at least partially formed extracellularly and are typically disc- or dome-shaped and are formed of numerous, minute equidimensional, calcite crystallites of simple shape (Siesser & Winter 1994, Young et al. 2005). These two types of calcification are typically attributed to the diploid and the haploid phases respectively (Fig. 7) by extrapolation from species from various families for which ploidy has been experimentally determined in culture (de Vargas & Probert 2004, Houdan et al. 2004, Young et al. 2005, de Vargas et al. 2007). Within this type of life cycle, the diploid phase can be non-motile (e.g. *Coccolithus pelagicus*) or motile (e.g. *Helicosphaera carteri*), whereas the haploid phases are exclusively motile.

Within known extant coccolithophore diversity, holococcolith calcification is the most common biomineralization mode in the haploid phase, being present in around eighty 'morphospecies'. This contrasts with the more than one hundred heteroccolithophores described within the clades for which an association with a HOL phase is expected (Young et al. 2003). In these terms, even though approximately thirty HET and HOL entities have already been linked within life cycles, there are still a large number of missing associations that remain to be found. This issue is discussed further in Chapter 4. Moreover, it means that there are probably several heterococcolithophores that may have unknown (rare) or non-calcified phases that remain hidden from classical microscopy methods. In this context, during this thesis a new method was developed, based on fluorescent *in situ* hybridization, that allows detection of non-calcified species/phases from water samples and would also be useful for linking uncoupled heteroccolithophores and holoccolithophores (Chapter 2).

Heterococcolithophore- Aragonitic coccolith phase

Another type of life cycle is the association of an HET phase with a phase bearing coccoliths made of aragonite, instead of calcite as in the majority of the species. This life cycle combination is unique to the heterococcolithophore genera *Alisphaera* and *Canistrolithus* (Fig. 6, Alisphaeraceae), which alternate with various types of *Polycrater* (Cros et al. 2000). It has been suggested that the aragonitic liths produced by this *Polycrater* stage may replace, for these species, the holococcoliths (Cros et al. 2000) and therefore that this probably represents the haploid phase and the HET phase should represent the diploid

phase. It is thought that these species probably evolved from an ancient group in which the haploid phase was not calcified, but this assumption needs to be tested in the future by phylogenetic analyses (Young et al. 2005).

Heterococcolithophore- Nannolith phase

Another relatively rare life cycle type consists of the association of an HET phase and a phase bearing nannoliths (an heterogeneous group of calcareous scales that includes a wide range of shapes). This is the case in the family Ceratolithaceae (Fig. 6). A complex association has been reported between the HET *Neosphaera coccolithomorpha*, the nannolith phase *Ceratolithus cristatus* which features a single large horseshoe-shaped nannolith (ceratolith), and a coccosphere of delicate hoop-shaped heterocccoliths (Alcober & Jordan 1997, Cros et al. 2000, Sprengel & Young 2000, Cros 2002). According to Young et al. (Young et al. 1998), the simplest hypothesis to explain this association is that the ceratoliths are equivalent to holococcoliths and represent the haploid phase and the *N. coccolithomorpha* phase are normal HET produced in the diploid phase, with the hoop-shaped coccoliths being alternative morphotypes produced by the same coccolith formation mechanism as the heterococcoliths. Once again there is currently no phylogenetic data that allows further interpretation of the origins of this life cycle association.

Another interesting nannolith-bearing coccolithophore is *Braarudosphaera bigelowii* (Fig. 6). Following the logic applied to the Ceratolithaceae, *B. bigelowii* should be the haploid phase, but unfortunately there are currently no indications of the life cycle counter-part. It would be, however, interesting to unveil this association since according to recent phylogenies this species belongs to a basal lineage within the Calcihaptophycideae (Takano et al. 2006), and this information would therefore help to address questions related to origins and mode of the calcification of the haploid and diploid phases.

Heterococcolithophore- non-calcifying phase

Historically, the life cycle type involving the association of a HET and a non-calcified phase are the best documented and most thoroughly studied cases, mainly because it is characteristic of most of the coastal (littoral) species of coccolithophore that are easier to access (Gayral & Fresnel 1983, Fresnel 1989, Billard 1994). Within this life cycle type, the diploid generation always consists of a HET phase, motile or not, whereas the haploid phase can be, depending on the species, pelagic and motile like in *Emiliania huxleyi* (Noelaerhabdaceae) (this life cycle will be described separately in the next section) or benthic,

forming pseudofilaments (*Pleurochrysis*, the *Apistonema*-stage), packets of cells (*Hymenomonas*) or palmelloid aggregations embedded in mucilage (*Ochrosphaera*).

This life cycle association has been confirmed by observations of syngamy and meiosis (*Pleurochrysis*) and by chromosome counts of stained nuclei (*Hymenomonas*, *Ochrosphaera*), and constituted one of the fundamental sources of information that Billard (1994) used to formulate her hypothesis.

It is also important to note that all of the cited species (except the Noelaerhabdaceae), are phylogenetically closely related to oceanic species that calcify in the haploid phase (holococolithophores) (Fig. 6). Therefore, the absence of holoccoliths in the haploid phase has been interpreted as a secondary loss, probably linked with the colonization of coastal environments (Young et al. 2005, de Vargas et al. 2007).

Finally, there are some coastal species such as *Isochrysis galbana*, phylogenetically close to the Noelaerhabdaceae, in which calcification has never been observed and for which a life cycle counter-part has never been reported. Possible hypotheses to explain these cases posit that these species may have lost calcification in both phases or instead that they preserved only the haploid phase and became asexual (Young et al. 2005, de Vargas et al. 2007).

C 1. Eco-Physiology of Calcihaptophycidae life cycles

Presently there are two reports of studies comparing the life cycle phases of coccolithophore species in terms of the physiological responses to abiotic factors. A third study on *E. huxleyi* will be exposed in the next section.

Through chemical and physical manipulation of the culture media, Noel et al. (Noel et al. 2004) were able (for the first time) to induce a life cycle change in a coccolithophore (*Calyptrosphaera sphaeroidea*, holococcolithophore) and unveiled its previously unknown diploid HET phase. Based on these results, it was proposed that the alternation of *Calyptrosphaera* life phases is regulated by changes between pelagic and coastal environments coupled with changes in seasonal conditions.

Houdan et al. (Houdan et al. 2006) conducted culture experiments to compare life cycle phases of two oceanic species, *Coccolithus braarudii* and *Calcidiscus leptoporus*, and showed that diploid and haploid phases responded differently to given nutrient conditions, diploids being more competitive in nutrient-rich media than haploids. In the other hand, the haploid phase of *Coccolithus braarudii* was shown to have the ability to ingest food-particles

(phagotrophy), confirming previous observations (Parke & Adams 1960). In addition, haploids were generally more sensitive to turbulence. These results led the authors to integrate their observations into Margalef's phytoplankton niche model (Margalef 1978) (Fig. 8), suggesting that the haploid phases of these species could be adapted to more stable and oligotrophic conditions and therefore would be more K-selected [2], whereas the diploid phases would flourish in more eutrophic and turbulent regions and would be more r-selected.

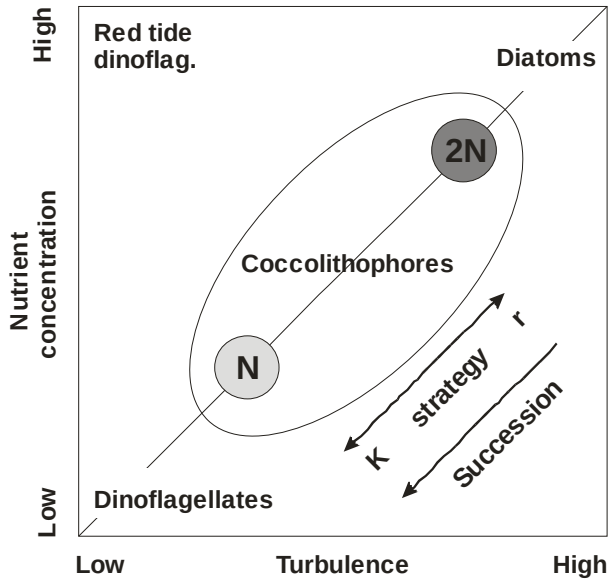


Figure 8. Modified version of the Margalef's two-dimensional niche space model, integrating the life cycle phases of coccolithophores (diploid (2n) and haploid (n)) (based on (Margalef 1978, Houdan et al. 2006).

The experimental work of Houdan et al (2006) is to some extent supported by field observations of both life cycle stages within the species *Coccolithus pelagicus* s.l. (Cachão & Moita 2000), in which the diploid phase was assigned to cold, nutrient-rich waters where advection and other mixing forces are strong, whereas the haploid phase was shown to be located in more oligotrophic areas.

Based on field observations, this model was recently applied to other coccolithophore species (*Helicosphaera carteri*, *Syracosphaera pulchra*, *Coronosphaera mediterranea*) in which both phases are found in the same water column. For these species, the diploid HET phase is generally present in relatively nutrient-rich deeper waters, whilst the haploid HOL

[2] In stable or predictable environments K-selection predominates, as the ability to compete successfully for limited resources is crucial, and populations of K-selected organisms typically are very constant and close to the maximum that the environment can bear. In unstable or unpredictable environments r-selection predominates, as the ability to reproduce quickly is crucial, and there is little advantage in adaptations that permit successful competition with other organisms, because the environment is likely to change again (based on Margalef, 1978).

phase is present in well-irradiated and relatively nutrient-poor surface waters (Cros 2002). These findings clearly demonstrate the existence of a spatial separation between two phases of the same species and indicate that each phase might be exploiting different niches (Fig. 9). Other studies have demonstrated the same pattern of separation, but over temporal rather than spatial scales. This was the case for *Coccolithus pelagicus*, in which both phases seem to inter-change their relative dominace over seasons (Okada & McIntyre 1977, Balestra et al. 2004).

In summary, it seems more and more apparent that the haplo-diplontic life cycle of coccolithophores is a key ecological strategy to exploit discrete niches and therefore survive in unstable environments or exploit different trophic regimes. Moreover, this strategy may be inherited from the non-calcified ancestor (Prymnesiophyceae) of coccolithophores and it is probable that coccolithophores have exploited the same ecological strategy throughout their evolutionary history, since fossil heterococcoliths and holococcoliths can be traced back down to the Triassic and Jurassic, respectively (Young et al. 2005). Nevertheless, more studies are needed to generalize this model to all coccolithophores, or conversely possibly to detect exceptions to this scheme.

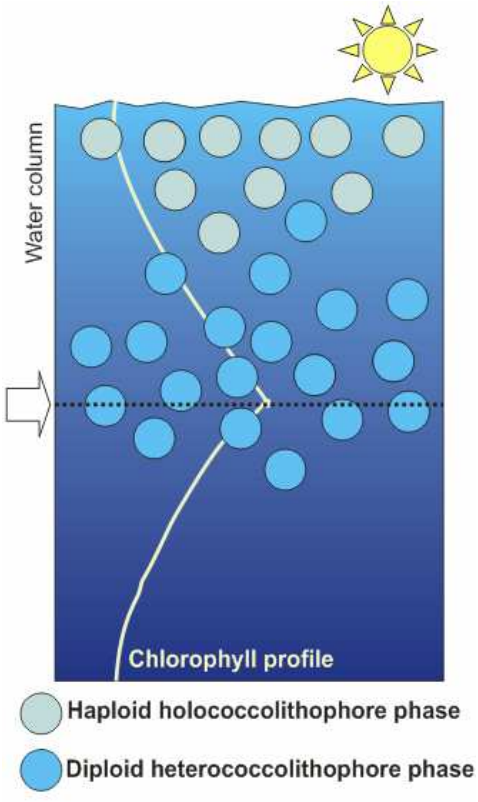


Figure 9. Schematic illustration of the vertical distribution of the haploid and diploid phases of *Helicosphaera carteri*. The arrow and dashed line represent the deep maximum of chlorophyll (DCM) (based on (Cros 2002)).

D. The particular case of Emiliania huxleyi

E. huxleyi is by far the most intensively studied coccolithophore due to its ubiquity throughout the oceans and to the production of extensive blooms that cover vast oceanic and neritic areas (Fig. 10) and which play an important role in the global environment (see Fig. 4) (e.g. (Holligan et al. 1993, Westbroek et al. 1993, Rost & Riebesell 2004, Tyrrell & Merico 2004, Iglesias-Rodriguez et al. 2008)).

Figure 10. Satellite image of an *Emiliania huxleyi* bloom in the entry of the English Channel off Brittany (France) (source, *Emiliania huxleyi* home page: http://www.soes.soton.ac.uk/staff/tt/).

The life cycle of *E. huxleyi*, like in other prymnesiophytes, is haplo-diplontic and heteromorphic. Early studies by Klaveness (Klaveness 1972) and Green et al. (Green et al. 1996) performed *in vitro* with cultures, described the life cycle of *E. huxleyi* as being composed by three main phases (Fig. 11, schematic illustration of the hypothetical life cycle), all capable of independent mitotic multiplication: the bloom-producing non-motile diploid coccolith-bearing (HET) phase (also referred to as calcified or 'C-cell'), which alternates with a diploid non-calcified non-motile phase (also referred to as the 'naked cell' stage and sometimes considered a culture artefact and not part of the regular life cycle (Paasche 2001)), and finally the haploid non-calcifying biflagellated phase, bearing organic scales like the majority of the prymnesiophytes (also referred to as the scaly or 'S-cell' phase), and that probably serves at some point as gametes (Fig. 12).

All three life phases have been kept in culture for decades, but once again the *in situ* life cycle functioning and the mechanisms triggering the transition between phases are still unidentified (Laguna et al. 2001, Paasche 2001, Billard & Inouye 2004, Houdan et al. 2005). Indeed, even though the occasional appearance of haploid phase cells within diploid phase cultures has been observed several times ((Klaveness 1972, Paasche 2001) and our observations)), which gives a clear indication of an inherent (and expected) sexual behaviour in this species, neither meiosis nor syngamy have been observed.

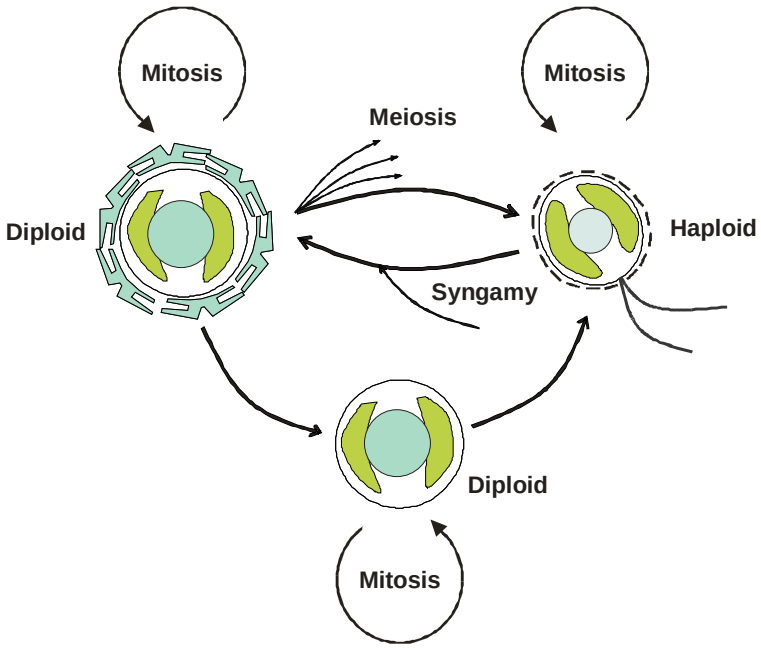


Figure 11. Schematic representation of the life cycle of *Emiliania huxleyi* suggested by Klaveness and Green (see text for details, illustration based on (Klaveness 1972, Green et al. 1996, Paasche 2001)).

There is, however, evidence from culture studies for a clear physiological differentiation between the various life forms in response to abiotic factors. These studies are particularly relevant when diploid calcified cells and haploid flagellated cells that were originally isolated from the same 'mother' clone are compared, thus minimizing the strain bias effect created when comparing clones of different origins. A first study of this type showed that diploid calcified phase cells and haploid cells have similar growth rates. However, the diploid calcified cells did not exhibit photoinhibition at irradiances up to 1000 µmol m^{-2} s^{-1}, whereas the haploid cells appear to be photoinhibited above 400-500 µmol m^{-2} s^{-1} (Houdan et al. 2005). This led to the suggestion that in nature the two phases may have similar potentials to grow and produce large populations but may exploit separate ecological niches (seasonal and/or spatial), similar to what has been documented for other coccolithophores (see above).

Nevertheless, all this studies focusing on this species were based solely in one phase of the life cycle of this species, the diploid calcified phase, which possess heterococcoliths and is therefore easily identifiable in marine water samples. In opposition nothing is known about the non-calcified (haploid or diploid phases). Using the innovative morpho-genetic methodology referred to above (Chapter 2), the *in situ* presence and ecological dynamics of both calcified and non-calcified *E. huxleyi* cells were for the first time studied in this thesis (Chapter 3). In complementary *ex situ* culture studies, the responses of both diploid and haploid phases of this species to a range of parameters were tested in order to understand the extent of eco-physiological segregation related to life cycling. These studies focussed on responses to both biotic parameters, i.e. viruses (Bratbak et al. 1993, Wilson et al. 2005) that are implicated in the termination of diploid phase blooms (see the following D1 section and Chapter 1), and on a range of abiotic environmental parameters, including investigation of intra-specific 'competition' between diploid and haploid phases (Annex 4).

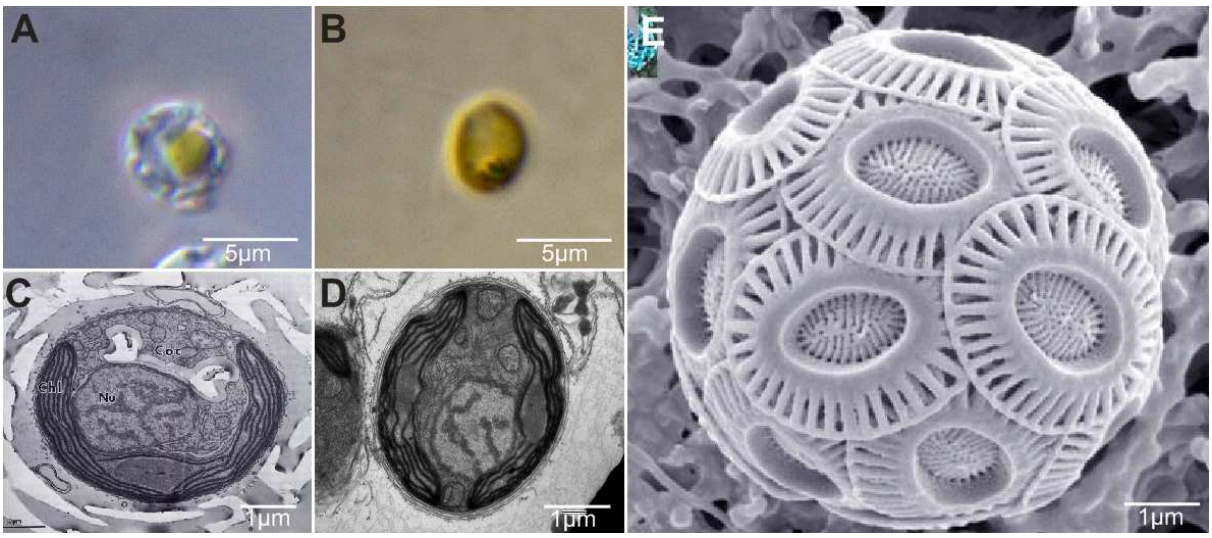


Figure 12. Microscopy images of *Emiliania huxleyi.*
A- Light micrograph of a diploid calcified phase cell; B- Light micrograph of a haploid phase cell (the two flagella are visible on the left side of the cell); C- TEM image of a thin section of a diploid calcified phase cell (the coccoliths around the cell and a coccolith being formed intracellularly are visible) (Pienaar 1994); D- TEM image of a thin section of a haploid phase cell (note the organic scales attached to the cell membrane); E- SEM image of a diploid calcified phase cell (C. Sprengel and J. Young, PlanktonNet).

D1. *Emiliania huxleyi* viruses and its impact of *E. huxleyi* populations

Viruses from the marine plankton, or virioplankton, are the most abundant and diverse biological entities in the oceans and possibly on Earth (Suttle 2005, 2007). Virtually all marine biota are infected and potentially killed by specific viruses, including metazoans

(Renault 2006, Smail & Egglestone 2006), but also unicellular protists and prokaryotes (Wommack & Colwell 2000, Van Etten et al. 2002, Boras et al. 2009). However, the ecological implications of pelagic virsues goes far beyond the mortality of their hosts. Through cell lysis and the release of intracellular contents to the water column, they substantially influence plankton community structures (Castberg et al. 2001, Larsen et al. 2001) and thus significantly alter nutrients and biogeochemical cycling (Wilhelm & Suttle 1999).

Viruses are known to infect and kill all the major groups of eukaryotic phytoplankton (Van Etten et al. 2002, Brussaard 2004), in particular during bloom events (Tarutani et al. 2000, Brussaard et al. 2005). This is especially true for the diploid calcified phase of *E. huxleyi* (Bratbak et al. 1993, Jacquet et al. 2002, Wilson et al. 2002, Schroeder et al. 2003, Martinez et al. 2007) (Fig. 13).

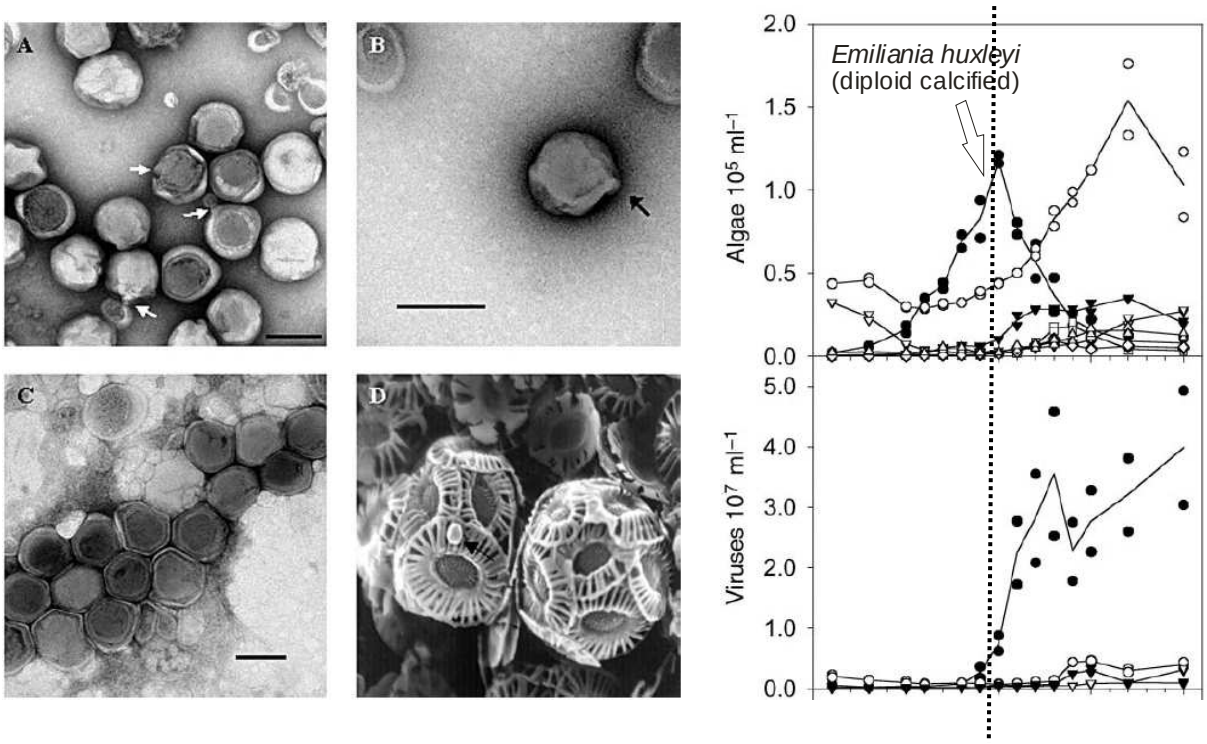


Figure 13. *Emiliania huxleyi viruses* (*Eh*V) and their impact on *E.huxleyi* blooms collapse
(A), (B) and (C) Transmission electron microscope images of *Eh*V, the arrows indicate possible tail stubs that may be involved in attachement (note also a defined capside layer surrounding the viruses interior); (D) Scanning electron microscope image of *Eh*V (arrowed) attached to an *E. huxleyi* cell. Scale bars = A-C, ~190 nm; D, no scale information available (Wilson et al. 2002). (Right) Exponential growth of various microalgae including *E. huxleyi* (top) and its viruses (below) determined by flow cytometry in a mesocosm enclosure (modified from (Castberg et al. 2001))

Many studies have shown that virus numbers typically increase following the demise of *E. huxleyi* blooms and are intrinsically linked to the decline of *E. huxleyi* large populations (Fig. 13, left). These viruses have been shown to be specific to *E. huxleyi* and therefore

referred to as *Emiliania huxleyi* viruses (or *Eh*Vs). *EhVs* belong to the family phycodnaviridae whose members contain double strand DNA (dsDNA) viruses which replicate completely or partially in the cytoplasm of the hosts (nucleocytoplasmic large DNA virsues - NCLDVs). *Eh*Vs are giant viruses ranging from 170 to 200 nm in diameter and have an icosahedral capside (Schroeder et al. 2002) enclosing a large circular genomes of ~400 kbase pairs. The 472 predicted genes from EhV86 are all transcribed during infections (Wilson et al. 2005).

In this thesis I explore the *in vitro* response of *E. huxleyi* diploid and haploid stages to *Eh*Vs (Chapter 1), in order to understand how the species as a whole deal with this major biotic impact threatening its survival.

3. References

Alcober J, Jordan RW (1997) An interesting association between *Neosphaera coccolithomorpha* and *Ceratolithus cristatus* (Haptophyta). European Journal of Phycology 32:91-93

Balch WM, Holligan PM, Ackleson SG, Voss KJ (1991) Biological and optical properties of mesoscale coccolithophore blooms in the Gulf of Maine. Limnol Oceanogr 36:692-643

Baldauf SL (2003) The deep roots of eukaryotes. Science 300:1703-1706

Balestra B, Ziveri P, Monechi S, Troelstra S (2004) Coccolithophorids from the southeast Greenland margin (northern north atlantic): production ecology and the surface sediment record. Journal of Nannoplankton Research 50:23-34

Baudoux A-C, Brussaard CPD (2005) Characterization of different viruses infecting the marine harmful algal bloom species *Phaeocystis globosa*. Virology 341:80-90

Bell G (1982) The masterpiece of nature: the evolution and genetics of sexuality, Vol, Berkeley

Billard C (1994) Life cycles. In: Green JC, Leadbeater BSC (eds) The Haptophyta Algae, Vol 51. Clarendon Press, Oxford, p 167-186

Billard C, Inouye I (2004) What's new in coccolithophore biology ? In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Boras JA, Sala MM, Vázquez-Domínguez E, Weinbauer MG, Vaqué D (2009) Annual changes of bacterial mortality due to viruses and protists in an oligotrophic coastal environment (NW Mediterranean). Environmental Microbiology 9999

Bown P, Young J (1998) Calcareous nannofossil biostratigraphy, Vol. Kluwer Academic Publishers, Cambridge

Bratbak G, Egge J, Heldal M (1993) Viral mortality of the marine alga *Emiliania huxelyi (*Haptophyceae) and the termination of the algal bloom. Marine Ecology Progess Series 93:39-48

Brownlee C, Nimer N, Dong LF, Merrett MJ (1994) Cellular regulation during calcification in *Emiliania huxleyi*. In: Green JC, Leadbeater BSC (eds) The Haptophyta Algae, Vol Special volume No. 51. The Systematics Association, Oxford, p 133-166

Brussaard CPD (2004) Viral control of phytoplankton populations - a review. The Journal of Eukaryotic Microbiology 51:125-138

Brussaard CPD, Kuipers B, Veldhuis MJW (2005) A mesocosm study of *Phaeocystis globosa* population dynamics I. Regulatory role of viruses in bloom control. Harmful Algae 4:859-874

Burki F, Shalchian-Tabrizi K, Minge M, Skjaveland A, Nikolaev SI, Jakobsen KS, Pawlowski J (2007) Phylogenomics Reshuffles the Eukaryotic Supergroups. PLoS ONE 2:e790

Cachão M, Moita MT (2000) *Coccolithus pelagicus*, a Productivity Proxy Related to Moderate Fronts off Western Iberia. Marine Micropaleontology 39:131-155

Castberg T, Larsen A, Sandaa RA, Brussard CPD, Egge JK, Heldal M, Thyrhaug R, van Hannen EJ, Bratbak G (2001) Microbial population dynamics and diversity during a bloom of the marine coccolithophorid Emiliania huxleyi (Haptophyta). Marine Ecology Progress Series 221:39-46

Cavalier-Smith T (1978) Nuclear volume control by nucleoskeletal DNA, selection for cell volume and cell growth rate, and the solution of the DNA C-value paradox. Journal Cellular Sciences 34:247-278

Cavalier-Smith T (1994) Origin and relationship of Haptophyta. In: Green JC, Leadbeater BSC (eds) The Haptophyta Algae, Vol Special volume No. 51. The Systematics Association, Oxford, p 413-435

Cavalier-Smith T (2002) Origins of the machinery of recombination and sex. Heredity 88:125-141

Cavalier-Smith T, Allsopp MTEP, Hauber MM, Gothe G, Chao EE, Couch JA, Maier U-G (1996) Chromobiote phylogeny: the enigmatic alga *Reticulosphaera japonensis* is an aberrant haptophyte, not a heterokont. European Journal of Phycology 31:255-263

Coelho SM, Peters AF, Charrier B, Roze D, Destombe C, Valero M, Cock JM (2007) Complex life cycles of multicellular eukaryotes: New approaches based on the use of model organisms. Gene 406:152-170

Cortes MY (2000) Further evidence for the heterococcolith-holococcolith combination Calcidiscus leptoporus-Crystallolithus rigidus. Marine Micropaleontology 39:35-37

Cros L (2002) Planktonic Coccolithophores of the NW Mediterranean., Universitat de Barcelona

Cros L, Kleijne A, Zeltner A, Billard C, Young JR (2000) New examples of holococcolith-heterococcolith combination coccospheres and their implications for coccolithophorid biology. Marine Micropaleontology 39:1-34

de Reviers B (2002) Biologie et phylogénie des algues, Vol Tome 1, Paris

de Reviers B (2003) Biologie et phylogénie des algues, Vol Tome 2, Paris

de Vargas C, Aubry M-P, Probert I, Young J (2007) Origin and evolution of coccolithophores: from coastal hunters to oceanic farmers. In: Falkowski P, Knoll AH (eds) Evolution of Aquatic Photoautotrophs. Elsevier Academic Press, New York, p 251-285

de Vargas C, Probert I (2004) New keys to the past: current and future DNA studies in coccolithophores. Micropaleontology 50:45-54

Destombe C, Godin J, LeFebvre C, Dehorter O, Vernet P (1992) Difference in dispersal abilities of haploid and diploid spores of Gracilaria verrucosa (Gracilariales, Rhodophyta). Botanica Marina 35:93-98

Edvardsen B (2001) Life history of Haptophytes. In: Esther G, Zingone A, Montresor M, Reguera B, Dale B (eds) LIFEHAB: life histories of microbial species causing harmful blooms. European Commission Directorate General Science, Research and Development, Majorca, Balearic Islands, Spain

Edvardsen B, Eikrem W, Green JC, Andersen RA, Moon-van der Staay SY, Medlin L, K. (2000) Phylogentic reconstruction of the Haptophyta inferred from the 18S ribosomal DNA sequences and available morphological data. Phycologia 39:19-35

Edvardsen B, Vaulot D (1996) Ploidy analysis of the two motile forms of *Chrysochromulina polylepsis* (Prymnesiophyceae). Journal of Phycology 32:94-102

Fresnel J (1989) Les coccolithophorides (Prymnesiophyceae) du littoral. Genres: Cricosphaera, Pleurochrysis, Cruciplacolithus, Hymenomonas et Ochrosphaera. Ultrastrucutre, cycle biologique, systématique. Univerité de Caen

Gast RJ, Mcdonnell TA, Caron DA (2000) srDNA-based taxonomic affinities of algal symbionts from a planktonic foraminifer and a solitary radiolarian. Journal of Phycology 36:172-177

Gayral P, Fresnel J (1983) Description, sexualité et cycle de développement d'une nouvelle Coccolithophoracée (Prymnesiophyceae): *Pleurochrysis pseudoroscoffensis* sp. nov. . Protistologica 19:245-261

Geisen M, Billard C, A.T.C. B, Cros L, Probert I, Young J (2002) Life cycle associations involving pairs of holococcolithophorids species: Intraspecific variation or cryptic speciation? European Journal of Phycology 37:531-550

Green JC, Course PA, Tarran GA (1996) The life-cycle of *Emiliania huxleyi*: A brief review and a study of relative ploidy levels analysed by flow cytometry. J Mar Syst 9:33-44

Green JC, Hibberd DJ, Pienaar RN (1982) The taxonomy of *Prymnesium* (Prymnesiophyceae including a description of a new cosmopolitan species, *P. patellifera* sp. nov., and further observations on *P. parvum* N. Carter. British Phycological Journal 17:363-382

Hackett J, Yoon H, Li S, Reyes-Prieto A, Rümmele SE, Bhattacharya D (2007) Phylogenomic analysis supports the monophyly of cryptophytes and haptophytes and the association of rhizaria with chromalveolates. Molecular Biology and Evolution 24:1702-1713

Hoekstra RF (2005) Why sex is good. Nature 434:571-573

Holligan PM, Fernandez E, Aiken J, Balch WM, Boyd P, Burkill PH, Finch M, Groom SB, Malin G, Muller K, Purdie DA, Robinson C, Trees CC, Turner SM, Van der Wal P (1993) A biogeochemical study of the coccolithophore Emiliania huxleyi, in the north atlantic. Global Biogeochemical Cycles 7:879-900

Holligan PM, Viollier M, Harbour DS, Camus P, Champagne-Philippe M (1983) Satellite and ship studies of coccolithophore production along a continental shelf edge. Nature 304:339-342

Houdan A, Billard C, Marie D, Not F, Saez A, Young G, Probert I (2004) Holococcolithophores-heterococcolithophores (Haptophyta) life cycles: flow cytometry analysis of relative ploidy levels. Systematics and Biodiversity 1:453-465

Houdan A, Probert I, Van Lenning K, Lefebvre S (2005) Comparison of photosynthetic responses in diploid and haploid life-cycle phases of *Emiliania huxleyi* (Prymnesiophyceae). Marine Ecology Progress Series 292:139-146

Houdan A, Probert I, Zatylny C, Véron B, Billard C (2006) Ecology of oceanic coccolithophores. I. nutritional preferences of the two stages in the life cycle of *Coccolithus braarudii* and *Calcidiscus leptoporus*. Aquatic Microbial Ecology 44:291-301

Hughes JS, Otto SP (1999) Ecology and the evolution of biphasic life cycles. The American Naturalist 154:306-320

Hurst LD, Peck JR (1996) Recent advances in understanding of the evolution and maintenance of sex. Tree 11:46-52

Iglesias-Rodriguez MD, Halloran PR, Rickaby REM, Hall IR, Colmenero-Hidalgo E, Gittins JR, Green DRH, Tyrrell T, Gibbs SJ, von Dassow P, Rehm E, Armbrust EV,

Boessenkool KP (2008) Phytoplankton calcification in a high-CO_2 world. Science 320:336-340

Inouye I, Kawachi M (1994) The haptonema. In: Green JC, Leadbeater BSC (eds) The Haptophyta Algae, Vol Special volume No. 51. The Systematics Association, Oxford, p 73-89

Jacobsen A (2002) Morphology, relative DNA content and hypothetical life cycle of *Phaeocystis pouchetii* (Prymnesiophycea) with special emphasis on the flagellated cell type. Sarsia 87:338-349

Jacquet S, Heldal M, Iglesias-Rodriguez D, Larsen A, Wilson W, Bratbak G (2002) Flow cytometric analysis of an *Emiliana huxleyi* bloom terminated by viral infection. Aquatic microbial ecology 27:111-124

Jeffrey SW, Wright JC (1994) Photosynthetic pigments in the Haptophyta. In: Green JC, Leadbeater BSC (eds) The Haptophyta Algae, Vol Special volume No. 51. The Systematics Association, Oxford, p 111-132

Jordan R, Cros L, Young J (2004) A revised classification scheme for living haptophytes. Journal of Nannoplankton Research 50:55-79

Jordan R, Kleijne A (1994) A classification system for living coccolithophores. In: Winter A, Siesser WG (eds) Coccolithophores. Cambridge Univerity Press, Cambridge, p 83-105

Jordan RW, Chamberlain AHL (1997) Biodiversity among haptophyte algae. Biodiversity and Conservation 6:131-152

Jordan RW, Kleijne A, Heimdal BR, Green JC (1995) A glossary of the extant Haptophyta of the world. Journal of the Marine Biological Association of the United Kingdom 75:769-814

Klaveness D (1972) Coccolithus huxleyi (Lohm.) Kamptn. II. The flagellate cell. aberrant cell types, vegetative propagation and life cycles. British Phycological Journal 7:309-318

Kleijne A (1991) Holococcolithophorids from the Indian Ocean, Res Sea, Mediterranean Sea and North Atlantic Ocean. Marine Micropaleontology 17:1-76

Kondrashov AS (1997) Evolutionary genetics of life cycles. Annual Review of Ecology and Systematics 28:391-435

Laguna R, Romo J, Read BA, Wahlund TM (2001) Induction of phase variation events in the life cycle of the marine coccolithophorid *Emiliania huxleyi*. Appl. Environ. Microbiol. 67:3824-3831

Larsen A, Bryant SSO (1998) Growth rate and toxicity of *Prymnesium parvum* and *Prymnesium patelliferum*(Haptophyta) in response to changes in salinity, light and temperature. Sarsia 83:409-418

Larsen A, Castberg T, Sandaa RA, Brussard CPD, Egge JK, Heldal M, Paulino A, Thyrhaug R, van Hannen EJ, Bratbak G (2001) Population dynamics and diversity of phytoplankton, bacteria and viruses in a seawater enclosure. Marine Ecology Progress Series 221:47-57

Larsen A, Edvardsen B (1998) A study of relative ploidy levels in *Prymnesium parvum* and *P. patelliferum* (Haptophyta) analysed by flow cytometry. Phycologia 37:412-424

Lee JJ (2006) Algal symbiosis in larger foraminifera. Symbiosis 42:63-75

Lewis WMJ (1985) Nutrient scarcity as an evolutionary cause of haploidy. American Naturalist 125:692-701

Mable BK, Otto SP (1998) The evolution of life cycles with haploid and diploid phases. BioEssays 20:453-462

Malin G, Liss P, Turner SM (1994) Dimethyl sulfide: production and atmospheric consequences. In: Green JC, Leadbeater BSC (eds) The Haptophyta Algae, Vol Special volume No. 51. The Systematics Association, Oxford, p 303-320

Malin G, Steinke M (2004) Dimethyl Sulfide Production: What is the Contribution of the Coccolithophores? In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Marchant HJ, Thompson LR (1994) Haptophytes in polar waters. In: Green JC, B.S.C. Leadbeater e (eds) The Haptophyta Algae, Vol Special volume No.51. The Systematics Association, Oxford

Margalef R (1978) Life-forms of phytoplankton as survival alternatives in an unstable environment. Oceanologica Acta 1:493-509

Martinez JM, Schroeder DC, Larsen A, Bratbak G, Wilson WH (2007) Molecular dynamics of *Emiliania huxleyi* and cooccurring viruses during two separate mesocosm studies. Appl. Environ. Microbiol. 73:554-562

Maynard Smith J, Szathmáry E (1999) The Origins of life, from the birth of life to the origin of language, Vol. Oxford University Press Inc, New York

Moestrup O (1994) Economic aspects: 'blooms', nuisance species, and toxins. In: Green JC, Leadbeater BSC (eds) The Haptophyta Algae, Vol Special volume No. 51. The Systematics Association, Oxford, p 265-285

Noel M-H, Kawachi M, Inouye I (2004) Induced dimorphic life cycle of a coccolithophorid, *Calyptrosphaera sphaeroidea* (Prymnesiophyceae, Haptophyta). Journal of Phycology 40:112-129

Not F, Massana R, Latasa M, Marie D, Colson C, Eikrem W, Pedrós-Alió C, Vaulot D, Simon N (2005) Late summer community composition and abundance of photosynthetic picoeukaryotes in Norwegian and Barents seas. Journal Limnology and Oceanography 50:1677-1686

Nuismer SL, Otto SP (2004) Host–parasite interactions and the evolution of ploidy. Proceeding of the National Academy of Sciences 101:11036-11039

Okada H, McIntyre A (1977) Modern coccolithophores of the Pacific and North Atlantic Oceans. Micropaleontology 23:1-55

Orr HA, Otto SP (1994) Does diploidy increase the rate of adaptation? Genetics 136:1475-1480

Otto SP, Lenormand T (2002) Resolving the paradox of sex and recombination. Nature Reviews Genetics 3:252-261

Paasche E (2001) A review of the coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae), with particular reference to growth, coccolith formation, and calcificationphotosynthesis interactions. Phycologia 40:503-529

Parke M, Adams I (1960) The motile (*Crystallolithus hyalinus* Gaarder and Markali) and non-motile phases in the life-history of *Coccolithus pelagicus* (Wallich). Journal of the Marine Biological Association of the United Kingdom 39:263-274

Peperzak L, Colijn F, Vrieling EG, Gieskes WWC, Peeters JCH (2000) Observations of flagellates in colonies of Phaeocystis globosa (Prymnesiophyceae); a hypothesis for their position in the life cycle. J. Plankton Res. 22:2181-2203

Perrot V (1994) Experimental approaches to the evolution of life cycles. Lectures of Mathematical Life Sciences 25:121-134

Pienaar RN (1994) Ultrastructure and calci®cation of coccolithophores. In: Winter A, Siesser WG (eds) Coccolithophores. Cambridge Univerity Press, Cambridge, p 13-37

Probert I, Fresnel J (2007) *Prymnesium lepailleurii* sp. nov. (Prymnesiophyceae), a new littoral flagellate from the Mediterranean Sea. European Journal of Phycology 42:289-294

Renault T (2006) Les virus infectant les bivalves marins. Virologie 10:35-41

Rost B, Riebesell U (2004) Coccolithophores calcification and the biological pump: reponse to environmental changes. In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Roth PH (1994) Distribution of coccoliths in oceanic sediments. In: Winter A, Siesser WG (eds) Coccolithophores. Cambridge University Press, Cambridge, p 199-218

Rousseau V, Chrétiennot-Dinet M-J, Jacobsen A, Verity P, Whipple S (2007) The life cycle of Phaeocystis : state of knowledge and presumptive role in ecology. Biogeochemistry 83:29-47

Schroeder DC, Oke J, Hall M, Malin G, Wilson WH (2003) Virus Succession Observed during an Emiliania huxleyi Bloom. Appl. Environ. Microbiol. 69:2484-2490

Schroeder DC, Oke J, Malin G, Wilson W (2002) Coccolithovirus (Phycodnaviridae): characterisation of a new large dsDNA algal virus that infects *Emiliana huxleyi*. Archives of Virology 147:1685-1698

Seoane S, Eikrem W, Edvardsen B (in press) *Prymnesium sol* sp. nov.(Haptophyta) - another prymnesiophyte with a dimorphic life cycle. Phycologia

Siesser WG, Winter A (1994) Composition and morphology of coccolithophore skeletons. In: Winter A, Siesser WG (eds) Coccolithophores, Vol 51-62. Cambridge University Press, Cambridge

Smail DA, Egglestone SI (2006) Virus infections of marine fish erythrocytes: electron microscopical studies on the blenny virus. Journal of Fish Diseases 3:47-54

Sprengel C, Young G (2000) First direct documentation of association of *Ceratolithus cristatus*, hoop-coccoliths and *Neosphaera coccolithomorpha* planoliths. Marine Micropaleontology 39:39-41

Steinmetz JC (1994) Sedimentation of coccolithophores. In: Winter A, Siesser WG (eds) Coccolithophores. Cambridge University Press, Cambridge, p 179-197

Suttle CA (2005) Viruses in the sea. Nature 437:356-361

Suttle CA (2007) Marine viruses - major players in the global ecosystem. Nat Rev Micro 5:801-812

Takano Y, Hagino K, Tanaka Y, Horiguchi T, Okada H (2006) Phylogenetic affinities of an enigmatic nannoplankton, Braarudosphaera bigelowii based on the SSU rDNA sequences. Marine Micropaleontology 60:145-156

Tarutani K, Nagasaki K, Yamaguchi M (2000) Viral impacts on total abundance and clonal composition of the harmful bloom-forming phytoplankton *Heterosigma akashiwo*. Appl. Environ. Microbiol. 66:4916-4920

Thomsen HA, Buck KR, Chavez FP (1994) Haptophytes as components of marine phytoplankton. In: Green JC, Leadbeater BSC (eds) The Haptophyta Algae, Vol Special volume No. 51. The Systematics Association, Oxford, p 187-208

Thomsen HA, Østergaard JB, Hansen LE (1991) Heteromorphic life histories in artic coccolithophorids (Prymnesiophyceae). Journal of Phycology 27:634-642

Thornber CS (2006) Functional Properties of the Isomorphic Biphasic Algal Life Cycle. Integrative and Comparative Biology 46:605-614

Tyrrell T, Merico A (2004) *Emiliania huxleyi*: bloom observation and the conditions that induce them. In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Valero M, Richerd S, Perrot V (1992) Evolution of alternation of haploid and diploid phases in life cycles. Trends in Ecology and Evolution 7:25-29

Van Den Hoek C, Mann DG, Jahns HM (1995) Algae: an introduction to phycology, Vol. Cambridge University Press, Cambridge

Van Etten JL, Graves MV, Müller DG, Boland W, Delaroque N (2002) Phycodnaviridae – large DNA algal viruses. Archives of virology 147:1479-1516

Vaulot D, Birrien J-L, Marie D, Casotti R, Veldhuis MJW, Kraay GW, Chretiennot-Dinet M-J (1994) Morphology, ploidy, pigment composition, and genome size of cultured strains of Phaeocystis (Prymnesiophyceae). Journal of Phycology 30:1022-1035

Westbroek P, Brown CW, Van Bleijswijk J, Brwonlee C, Brummer GJ, Cote M, Egge J, Fernandez E, Jordan R, Knappersbusch M, Steffels J, Veldhuis M, Van der Wal P, Young J (1993) A model system approach to biological climate forcing: the example of Emiliania huxleyi. Global Planet Change 8:27-46

Wilhelm SW, Suttle CA (1999) Viruses and nutrient cycles in the sea. Bioscience 49:781-788

Wilson WH, Schroeder D, Allen M, Holden MTG, Parkhill J, Barrell BG, Churcher C, Hamlin N, Mungall K, Norbertczak H, Quail MA, Price C, Rabbinowitsch E, Walker D, Craigon M, Roy D, Ghazal P (2005) Complete genome sequence and lytic phase transcription profile of a coccolithovirus. Science 309

Wilson WH, Tarran GA, Schroeder D, Cox M, Oke J, Malin G (2002) Isolation of viruses responsible for the demise of an *Emiliania huxleyi* bloom in the English Channel. J. Mar. Biol. Ass. U.K. 82:369-377

Winter A, Jordan RW, Roth PH (1994) Biogeography of living coccolithophores in ocean waters. In: Winter A, Siesser WG (eds) Coccolithophores. Cambridge University Press, Cambridge, p 161-177

Wommack KE, Colwell RR (2000) Virioplankton: viruses in aquatic ecosystems. Microbiol. Mol. Biol. Rev. 64:69-114

Yoon HS, Hackett JD, Ciniglia C, Pinto G, Bhattacharya D (2004) A molecular timeline for the origin of photosynthetic eukaryotes. Mol Biol Evol 21:809-818

Young G, Bown P (1997) Cenozoic calcareous nannoplakton. Journal of Nannoplankton Research 19

Young G, Jordan RW, Cros L (1998) Notes on nannoplankton systematics and life cycles—*Ceratolithus cristatus*, *Neosphaera coccolithomorpha* and *Umbilicosphaera sibogae*. Journal of Nannoplankton Research 20:89-99

Young J, Geisen M, Cros L, Kleijne A, Sprengel C, Probert I, Østergaard J (2003) A guide to extant coccolithophore taxonomy. Journal of Nannoplankton Research:125 pp.

Young JR, Didymus JM, Bown PR, Prins B, Mann S (1992) Crystal assembly and phylogenetic evolution in heterococcoliths. Nature 356:516-518

Young JR, Geisen M, Probert I (2005) A review of selected aspects of coccolithophore biology with implications for paleobiodiversity estimation. Micropaleontology 51:267-288

Young JR, Henriksen K (2003) Biomineralization within vesicles: The calcite of coccoliths. In: Biomineralization, Vol 54, p 189-215

3. Objectives and thesis contents

The principal aim of this thesis was to investigate the physiological differentiation and the ecological roles of the different life cycle phases of the ubiquitous coccolithophore *Emiliania huxleyi*. To achieve this a combination of laboratory and field approaches was employed to explore two principal issues:

- **Physiology:** Study of the responses of the diploid and haploid phases of *E. huxleyi* to specific viruses.
- **Ecology:** Exploration of the role of the different life cycle phases in nature.

To address the first objective a series a cross-infection assays was performed using multiple *E. huxleyi* (haploid and diploid) strains and *E. huxleyi* virus (*Eh*V) isolates (**Chapter 1**).

To address the second objective a new method was first developed, based on a combination of fluorescent *in situ* hybridization and cross-polarized microscopy (the COD-FISH method), to specifically visualize and quantify coccolithophores (including *E. huxleyi*) and differentiate calcified from non-calcified cells in seawater samples (**Chapter 2**).

This new method was then applied to samples from different marine environments, including an artificially induced bloom (mesocosm, Norway), two coastal environments, one off-shore Roscoff (France) and the other in a fjord south of Bergen (Norway), and one oceanic environment in the west Pacific (**Chapter 3**). Complementary laboratorial studies were performed to further interpret the results of field surveys (**Annex 4**)

Studies on life cycles of other haptophytes were also performed during this thesis, producing new information on diversity and evolution of this trait in this group.

In the second part of this manuscript, the discovery of the first life cycle combinations in the coccolithophore family Pontosphaeraceae are presented (**Chapter 4**). A preliminary study on the life cycle of the Pavlovophyceae, a group that diverged early in the history of the Haptophyta, is then presented (**Chapter 5**). The aim of this study was to shed light on the origin of the haptophyte life cycle strategy and thereby to assess the evolutionary significance of the haplo-diploidy and niche differentiation typically observed in the modern prymnesiophyte taxa.

PART II. ECO-PHYSIOLOGY OF *EMILIANIA HUXLEYI* LIFE CYCLE

CHAPTER 1. The "Cheshire Cat" escape strategy of the coccolithophore *Emiliania huxleyi* in response to viral infection

The "Cheshire Cat" escape strategy of the coccolithophore *Emiliania huxleyi* in response to viral infection

Miguel Frada[1,2]*, Ian Probert[1], Mike J. Allen[3], William H. Wilson[4], Colomban de Vargas[1]

[1]CNRS & Université Pierre et Marie Curie (UMR 7144), Equipe EPPO - Evolution du Plancton et Paléo-Océans, Station Biologique, 29682 Roscoff, France
[2]Departamento de Geologia, Faculdade de Ciências, Universidade de Lisboa, Edifício C6, Campo Grande, 1749-016 Lisboa, Portugal
[3]Plymouth Marine Laboratory, Prospect Place, The Hoe, Plymouth PL1 3DH, United Kingdom
[4]Bigelow Laboratory for Ocean Sciences, 180 McKown Point, P.O. Box 475, West Boothbay Harbor, ME 04575-0475

*corresponding author:
Miguel Frada
Station Biologique
Equipe EPPO - Evolution du Plancton et Paléo-Océans (UMR 7144)
Place Georges Teissier, Roscoff (France)
Phone : 33 (0) 2-98-29-25-28
email : frada@sb-roscoff.fr

Published in : **Proceedings of the National Academy of Sciences USA, 2008 vol.104 no.41 15944–15949**
Communicated by Paul G. Falkowski, Rutgers, The State University of New Jersey, New Brunswick, NJ, August 6, 2008

- **Annex 1**: commentary by Peter Morin (Rutgers University) about this paper

⎔ the structure of the manuscript submitted to Proceedings of the National Academy of Sciences USA were maintained

Abstract

The coccolithophore *Emiliania huxleyi* is one of the most successful eukaryotes in modern oceans. The two phases in its haplodiploid life cycle exhibit radically different phenotypes. The diploid calcified phase forms extensive blooms, which profoundly impact global biogeochemical equilibria. By contrast, the ecological role of the noncalcified haploid phase has been completely overlooked. Giant phycodnaviruses (*Emiliania huxleyi* viruses, *Eh*Vs) have been shown to infect and lyse diploid-phase cells and to be heavily implicated in the regulation of populations and the termination of blooms. Here, we demonstrate that the haploid phase of *E. huxleyi* is unrecognizable and therefore resistant to EhVs that kill the diploid phase. We further show that exposure of diploid *E. huxleyi* to EhVs induces transition to the haploid phase. Thus we have clearly demonstrated a drastic difference in viral susceptibility between life cycle stages with different ploidy levels in a unicellular eukaryote. Resistance of the haploid phase of *E. huxleyi* provides an escape mechanism that involves separation of meiosis from sexual fusion in time, thus ensuring that genes of dominant diploid clones are passed on to the next generation in a virus-free environment. These ‘‘Cheshire Cat’’ ecological dynamics release host evolution from pathogen pressure and thus can be seen as an opposite force to a classic ‘‘Red Queen’’ coevolutionary arms race. In *E. huxleyi*, this phenomenon can account for the fact that the selective balance is tilted toward the boom-and-bust scenario of optimization of both growth rates of calcifying *E. huxleyi* cells and infectivity of EhVs.

Keywords

Eukaryotic life cycle, haplo-diploidy, marine viruses, host-parasite interaction, red queen

1. Introduction

The coccolithophore *Emiliania huxleyi* (Lohmann) Hay and Mohler is one of the most abundant and widely distributed photosynthetic unicellular eukaryotes in modern oceans. Coccolithophores (Calcihaptophycidae, Haptophyta) produce composite skeletons of minute calcite platelets (the coccoliths) and, consequently, have been key contributors to both the oceanic carbon pump and the counterpump, and thus to the flux of CO_2 between atmosphere and oceans, since their origin in the Triassic (de Vargas et al. 2007). In this context, the impact of predicted anthropogenically induced ocean acidification on calcifying plankton is a subject of intense debate (Riebesell et al. 2000, Iglesias-Rodriguez et al. 2008). Coccolith-bearing *E. huxleyi* cells periodically develop extensive blooms covering wide coastal and midoceanic areas at high latitudes in both the northern and southern hemispheres. Termination of these blooms is accompanied by massive release of organic and inorganic matter to the water column, including detached coccoliths that reflect sunlight and are readily detectable in satellite images (Tyrrell & Merico 2004). Over the last decade, the role of large (~175 nm) lytic coccolithoviruses (Phycodnaviridae), named *E. huxleyi* viruses (EhVs), in the regulation and termination of massive *E. huxleyi* blooms has been clearly established (Wilson et al. 2002), to the extent that this system has become a case study in marine virology (e.g., refs. (Bratbak et al. 1993, Allen et al. 2005, Wilson et al. 2005)). Both *E. huxleyi* and EhV populations have been shown to be genetically diverse (Schroeder et al. 2002), with host succession suggested to follow ‘‘kill the winner’’ dynamics (Thingstad 2000). However, recent observations show that the same *E. huxleyi* genotype blooms and is infected and decimated by the same EhV genotype over multiannual time scales (Martinez et al. 2007).

What, then, is the selective advantage for the ‘‘winner’’ *E. huxleyi* clone(s) to bloom? Is there intense and permanent selection pressure for resistance to viral infection [‘‘Red Queen’’ (RQ) dynamics]? And how is the high lytic virulence of EhVs sustained? These questions are fundamental to understanding the evolutionary ecology of this biogeochemically important species. The answers may be related to sex and life cycling, basic biologic features of unicellular eukaryotes that are typically ignored in oceanographic models addressing the ecology of planktonic functional groups. The fitness of many eukaryotic species may be based on their potential for alternation between variable life cycle phases and adaptation of each phase to different ecological niches (Valero et al. 1992, Thornber 2006), including in terms of biological interactions. Current evidence suggests that coccolithophore life cycles are characterized by independent haploid and diploid phases displaying radically different morphologies (Billard 1994, Houdan et al. 2004) and distinct physiologies (refs. (Houdan et al.

2005, Houdan et al. 2006); our unpublished data). In *E. huxleyi*, the life cycle comprises two main forms: the diploid (2*N*), nonmotile, coccolith-bearing phase that forms blooms, and the haploid (*N*) flagellated phase that possesses nonmineralized organic scales overlying the cell membrane (Green et al. 1996, Paasche 2001). This motile, noncalcifying, haploid stage is not easily amenable to identification by conventional microscope techniques and has been almost completely overlooked by biological oceanographers. Its ecological role and the importance of sexual cycling in *E. huxleyi* in the natural environment remain unknown. Flow cytometric surveys of mesocosm blooms of 2*N E. huxleyi* have revealed the onset, after virus-mediated bloom demise, of new active populations of cells with the same chlorophyll fluorescence signature but lower light-scattering values than 2*N* calcified cells (Castberg et al. 2001, Jacquet et al. 2002). We hypothesized that these new populations consisted of noncalcifying *N* cells, and that their presence reflected their resistance to infection by the viruses responsible for the decline of the 2*N* blooms.

In the present work, we explore the *in vitro* infectivity profiles of both the coccolith-bearing diploid and noncalcifying haploid life cycle phases of *E. huxleyi* by using multiple host and viral strains and various experimental setups. The results show the critical significance of life cycling in the survival and ecological dynamics of *E. huxleyi*. They illustrate a previously unrecognized type of ecological and evolutionary interaction that opposes classical RQ host–pathogen dynamics and is potentially a fundamental force for the maintenance of sex and life cycling and for untying the constraints imposed by pathogens in the evolution of eukaryotic microbes.

2. Results

As a first approach, we qualitatively scanned the infectivity of the 15 available EhV strains against both life cycle stages of three different strains of *E. huxleyi* (RCC1216, RCC1249, and RCC1213). All 2*N E. huxleyi* strains were sensitive to five of the viral strains (EhV201, EhV202, EhV205, EhV207, and EhV208), whereas none of the *N* strains were affected. As no obvious differences in infectivity were observed between the *E. huxleyi* and EhV strains tested, the host–virus combination RCC1216:EhV201 was chosen for subsequent experiments.

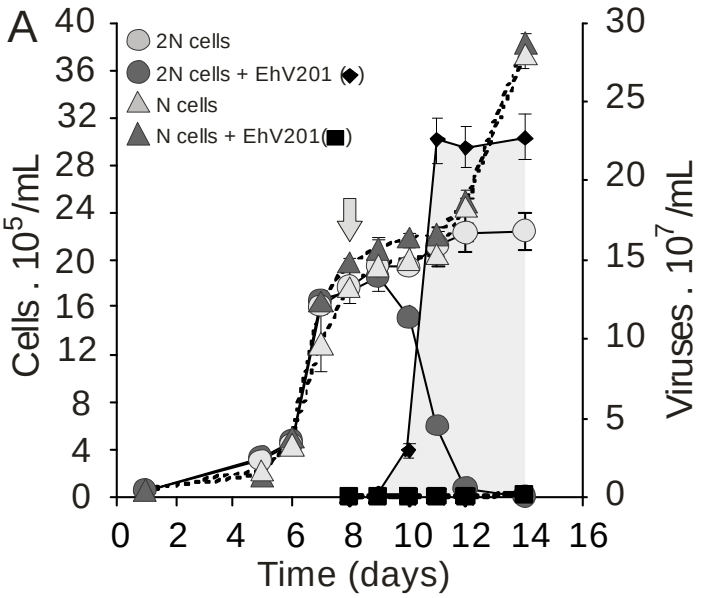


Figure 14. The impact of EhV on the growth of diploid and haploid *E. huxleyi*. Growth curves of both life cycle stages of *E. huxleyi* (strain RCC1216) and the virus EhV201 are shown. The arrow indicates the day of virus addition (multiplicity of infection 0.2). Standard deviation bars are generally too short to be visible.

We then monitored the growth (Fig. 14) and photosynthetic activity [supporting information (SI) Fig. S1] of both infected and noninfected *E. huxleyi* 2*N* and *N* phases. In 2*N* cultures, daily flow cytometry and fluorimetric measurements revealed 96% and 98% decreases of cell density and photosynthetic activity, respectively, within 3 days of virus inoculation into the growing culture. There was a concurrent increase in viral particle concentration from 3.5 x 10^5 to 23 x 10^7 viral particles per ml from days 8–14. In contrast, no differences were observed between infected and noninfected *N* cultures throughout the experiment, and virus concentration neither increased nor decreased. Visually, the 2*N* cultures became transparent, and deposits of cell debris were observed at the bottom of the culture flasks 3 days after infection (Fig. S2). To further verify the absence of infection of the *E. huxleyi* haploid phase, various viral densities and coexistence times were tested (Fig. S3). Haploid cultures that were previously used for infection experiments and fresh *N* cultures were incubated for 26 days with viruses at various multiplicities of infection (MOI). All cultures displayed similar growth curves, reaching typical concentrations of 15 x 10^5 to 20 x 10^5 cells per ml at day 10.

We then used transmission electron microscopy (TEM) and PCR of the gene coding for the EhV major capsid protein (MCP) (Fig. 15) to verify the absence of viral adsorption and production by haploid *E. huxleyi* suggested by flow cytometry. No viral capsids were detected inside or adsorbed to the *N* cells from virus-infected cultures in any of the multiple

TEM preparations, whereas capsids were obvious in the cytoplasm of infected 2*N* cells as early as 1 day after virus addition (Fig. 15*A*). Furthermore, the *MCP* gene was easily detected by PCR of DNA extracts from filtered and washed infected 2*N* cells, whereas the *N* cells in contact with viruses never yielded positive amplifications (Fig. 15*B*).

Finally, we set up a series of 50-day experiments to test longer-term responses of 2*N* and mixed 2*N*–*N* *E. huxleyi* cultures to viruses. The infected 2*N* cultures crashed 5 days after infection (Fig. 16*A*). On day 6, a small new peak of cells (~4 x 10^5 cells per ml) was observed within the flow cytometric window defined for *N* cells (Fig. S4).

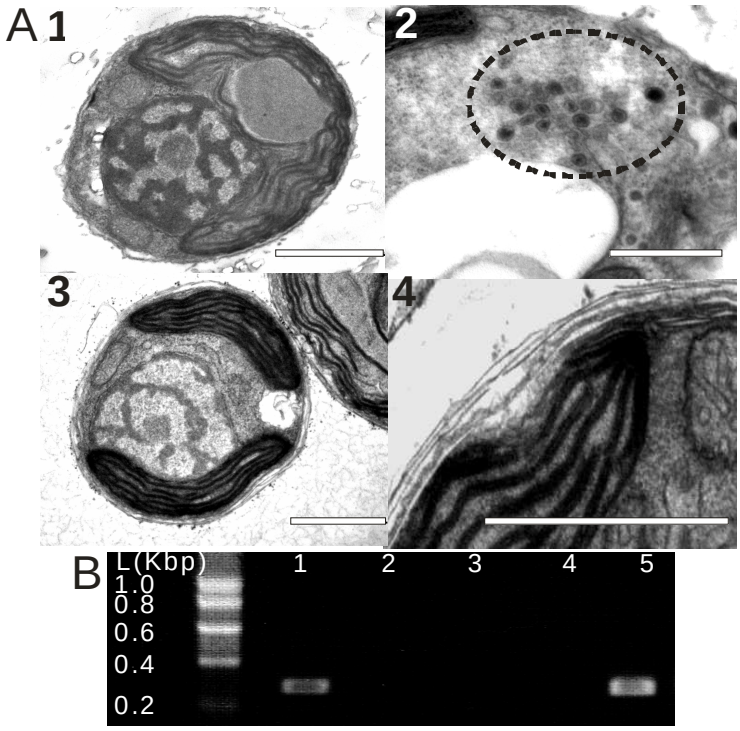


Figure 15. Microscopy and genetic tests for the presence/absence of EhVs inside and/or on infected *E. huxleyi* haploid and diploid cells. (*A*) TEM: 1, healthy 2*N* cell before infection; 2, 2*N* cell at day 1 after infection, displaying newly formed viral particles (dashed circle); 3, *N* cell from an infected culture; and 4, detail of the *N* cell periplast, showing the presence of organic scales attached to the membrane and the absence of coccoliths and viruses. (Scale bars: 1µm.) (*B*) PCR amplifications of the viral *MCP* gene at day 11 after infection (see Fig. 14). Agarose gel lanes: 1, positive control (EhV201 DNA extract); 2, *N* culture; 3, 2*N* culture; 4, *N* culture exposed to viruses; and 5, 2*N* culture exposed to viruses. Cells were carefully filtered and washed several times to remove free viral particles before DNA extractions.

Microscope observations revealed that these *E. huxleyi* cells possessed neither coccoliths nor flagella, resembling moribund noncalcified diploid cells. Thirty of these were sorted by flow cytometry into fresh, virus-free culture media, but none of them established a new growing population. Between days 7 and 23, cell density remained at a background level of 10 to 10^3 cells per ml. At day 24, however, a new population of *N* cells appeared, as

observed by both flow cytometry (Fig. 16*A*) and light microscopy. The concentration of these actively swimming *N* cells reached a plateau after ~10 days. In contrast, the noninfected 2*N* control culture declined after the plateau phase, with no sign of the presence of *N* cells (Fig. 16*B*). Two additional long-term experiments were performed with both life cycle stages mixed in the same culture vessel. In the first of these experiments, the diploid and haploid stages were grown together in the presence of viruses (Fig. 16*C*). The population dynamics were similar to those in the experiment with 2*N* cells only, with the same peak of drifting, noncalcified cells at day 6, followed by a lag phase of very low cell densities, and the development of the *N* population by day 20. The population of *N* cells attained a higher density than in the assay that started with 2*N* cells only. In the last experiment, 2*N* and *N* cells were grown together without viruses. The shape of the 2*N* growth curve was identical to that of the culture without *N* cells (Fig. 16*B*), although of lower cell density. However, haploid cell density remained low and constant (~10^4 cells per ml) during the first 24 days and then decreased to minimum values of ~10^3 cells per ml in the second half of the experiment. Controls with pure haploid cultures with or without viruses were conducted, and these displayed typical haploid cell growth curves (data not shown).

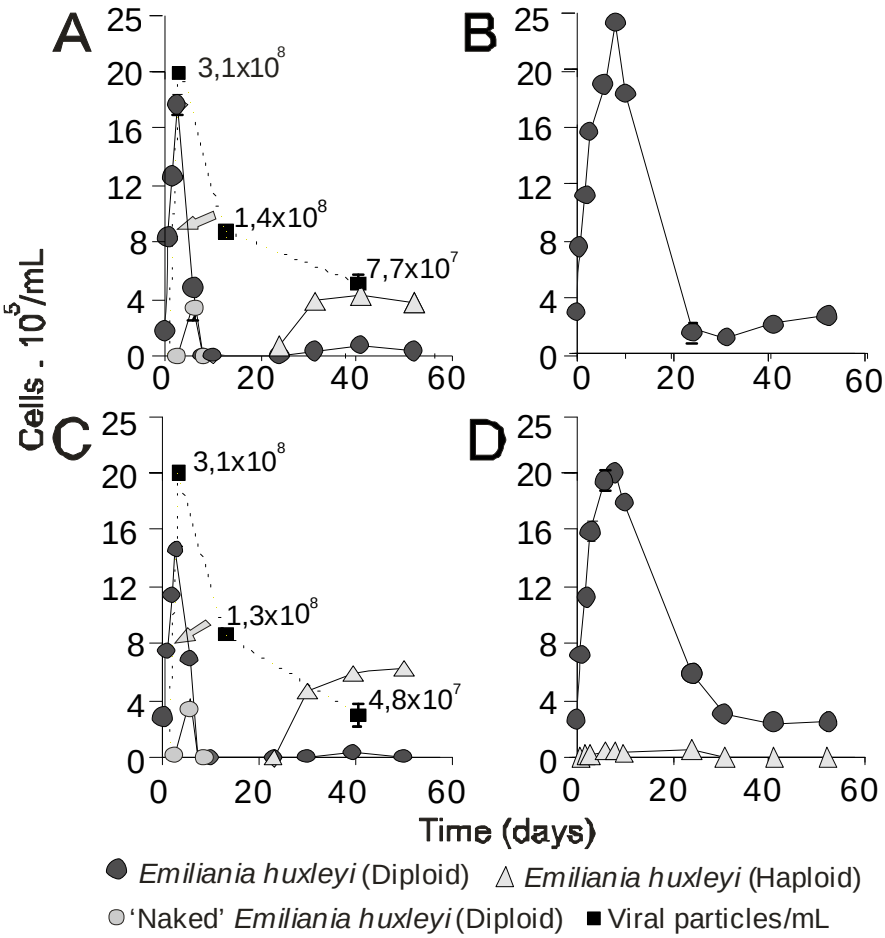


Figure 16. (previous page) Long-term infection assays of 2*N* and mixed 2*N*–*N* cultures of *E. huxleyi*. (*A*) 2*N* cells infected with EhV201 virus. (*B*) 2*N* cells without virus. (*C*) Mixture (100:1) of 2*N* and *N* cells infected with EhV201. (*D*) Mixture (100:1) of 2*N* and *N* cells without virus. 2*N* naked cells (due to coccolith loss after viral infection) were distinguished from *N* cells by their permanent immobility. The arrows indicate time of infection. Standard deviation bars are generally too short to be visible.

3. Discussion

Our data demonstrate that the noncalcifying haploid phase of the coccolithophore *E. huxleyi* is resistant to viruses that infect and lyse the diploid calcifying phase of the same species. This phenomenon was confirmed *in vitro* with multiple *E. huxleyi* and EhV strains. In all experiments using haploid *E. huxleyi* cultures, concentrations of free-floating viral particles were stable over time, and neither viral capsids nor viral DNA was detected by TEM or PCR (Fig. 15) within *N* cells in contact with viruses. This observation indicates the existence of a mechanism impeding the adsorption of viruses in *E. huxleyi* haploid cells. Differential susceptibility to viral infection has been reported between clones of the same ploidy level in a number of microalgal taxa (Tarutani et al. 2000), but there has been no previous clear demonstration of a drastic difference in viral susceptibility between life cycle stages with different ploidy levels in a marine protist. Our results further indicate that viral infection may trigger meiosis, or at least a shift from diploid to haploid populations, in *E. huxleyi* (Fig. 16). These observations have important implications for the ecology and evolution of both *E. huxleyi* and EhVs, and potentially all oceanic protists with sexual life cycles.

Mechanisms of Viral Resistance of E. huxleyi Haploid Cells

To initiate infection, viruses attach to host-specific cell surface receptors (Baranowski et al. 2001). Obvious phenotypic differences exist in the nature of the cell cover between life cycle phases in *E. huxleyi* (Fig. 15). One or several loose layers of interlocking coccoliths surround diploid cells, but these do not produce organic scales covering the cell membrane (commonly known as body scales and typical for most haptophytes). By contrast, haploid cells do not produce coccoliths, but their cell surface is covered by distinctively tightly packed body scales organized in overlapping layers (Green et al. 1996). This haploid cell covering, characteristic of most noncalcifying members of the order Isochrysidales, may efficiently prevent viruses from coming into contact with putative receptor sites on the cell membrane. Alternatively, plasmalemma molecules recognized by EhV capsids may be modified or simply absent in haploid cells. Modification or loss of receptor molecules is the most common way in which bacteria develop resistance to bacteriophages (Bohannan &

Lenski 2000), but these phenotypic differences typically result from mutation(s) of the genotype rather than differential gene expression, as would most likely be the case over the *E. huxleyi* life cycle. Note that the fact that the three haploid *E. huxleyi* strains tested proved resistant to viral infection makes it highly improbable that resistance results from a simple Mendelian allele segregation effect. The receptors to which viruses bind typically serve primary metabolic functions in the host, and hence receptor differences between life cycle phases would imply physiologic differentiation. In this context, a notable difference between *E. huxleyi* life cycle phases is calcium metabolism, which is known to be particularly intense in the calcifying 2*N* phase (Brownlee & Taylor 2004), but is presumably negligible in the noncalcifying *N* phase. Calcium often promotes the physical interactions between viruses and host receptors through a direct effect on the conformation of the viral capsid as, for example, in the hepatitis A virus in humans (Bishop & Anderson 1997). Other mechanisms of viral avoidance reported in marine protists include the extracellular production of viral inhibitors, like the cell wall sulfated polysaccharide produced by the red microalga *Porphyridium* sp. (Huheihel et al. 2002), the exudation of a protective extracellular polysaccharidic layer or aggregates that may trap viruses, as observed in the colony-forming haptophytes *Phaeocystis globosa* and *Phaeocystis pouchetii* (Jacobsen et al. 1996, Brussaard et al. 2005), or the secretion of dimethyl sulfide and acrylic acid, which are known to inhibit infection (Evans et al. 2006a). However, such mechanisms do not seem to be relevant in the case of the *E. huxleyi* life cycle, as EhVs infected 2*N* cells in mixed 2*N*–*N* cultures as efficiently as in pure 2*N* cultures.

Viral Infection and Life Cycle Phase Transition in E. huxleyi

Preliminary indications suggest that life cycle phase switches in coccolithophores may be regulated by chemical (Noel et al. 2004) or physical (Billard 1994) properties of the medium. Our data show that biotic interactions with viruses may also play a key role in directly triggering life cycle changes. Oxidative stress in response to viral infection could be the trigger for both the peak of dead *E. huxleyi* 2*N* cells at day 6 after infection and the sexual diploid-to-haploid transition observed in an initially pure 2*N* culture (Fig. 16*A*). As has commonly been observed in infected plant and animal cells, viral infection can induce elevated production of reactive oxygen species (ROS) in diploid *E. huxleyi* cultures (Evans et al. 2006b). ROS and viral infection were found to be associated with the induction of metacaspases and programmed cell death (PCD) in diploid *E. huxleyi* (Bidle et al. 2007), a phenomenon also demonstrated in other marine protists (Vardi et al. 1999). However, cell

death is not the exclusive response to oxidative stress in single-celled eukaryotes. ROS-induced PCD pathways are also clearly linked to the induction of life cycle phase transitions in phytoplankton. Cell cycle arrest and PCD are alternative responses to increased oxidative stress in the colonial green alga *Volvox carteri* (Nedelcu & Michod 2003), and developmental programs that lead to the concomitant formation of dead cells and spores have been described in protists, such as the slime mold *Dictyostelium discoideum*, as well as in several prokaryotes (Ameisen 2002). An alternative explanation could be that *N* cells are produced regularly by meiosis in 2*N* cultures, but remain at very low concentrations such that they are effectively undetectable by microscopy or flow cytometry. In our mixed 2*N*–*N* culture experiment (Fig. 16*D*), diploid cells clearly out-competed haploid cells to the point that the growth dynamics of 2*N* populations were strikingly similar with or without addition of *N* cells. In such a case, the very rare *N* cells would provide a permanent inoculum for the potential emergence of a haploid population, and viral infection would indirectly promote succession of life cycle stages through elimination of the more competitive diploid phase.

Ecological Implications

The visible worldwide ecological success of the diploid stage of *E. huxleyi* has stimulated interest in the underlying physiologic mechanisms that allow these calcifying cells to form extensive blooms under certain conditions (Tyrrell & Merico 2004). Two of the more remarkable capacities of *E. huxleyi* 2*N* cells are their exceptionally high phosphate uptake and the fact that they do not exhibit photoinhibition of photosynthesis, even at very high light intensities (ref. (Green et al. 1996) and references therein). These and other data help explain how the diploid *E. huxleyi* comes to dominate its phytoplankton competitors. However, in light of the growing body of evidence indicating the devastating impact of the highly infective EhVs on these blooms, the evolutionary advantages of this ecological strategy are not clear. Why has investment by *E. huxleyi* 2*N* of more resources into defense mechanisms at the expense of growth not been selected? Why has reduction of infectivity of EhVs to confer a clear selective advantage for blooming in their host clones not been selected? The ability of haploid cells to escape viral infection can explain why the selective balance is tilted so far toward optimization of growth rates and infectivity in *E. huxleyi* 2*N* cells and EhVs, respectively. By increasing massively in density as a result of successive mitoses, more cells of a given 2*N* clone are likely to undergo meiosis, potentially as a direct response to viral infection. If newly formed *N* cells were susceptible to viral infection they would be rapidly decimated, because viral density is highest during bloom demise (Wilson et al. 2002,

Martinez et al. 2007), and because motile cells are theoretically more likely to encounter viral particles (Brussaard 2004). Blooming would then have no selective advantage compared with a strategy of maintaining low background 2*N* cell concentrations. On the other hand, transformation into a haploid phenotype invisible to the virus provides an escape mechanism that ensures that the genes of an individual (or clone) are passed on to the next generation. It can be argued, therefore, that this phenomenon dictates positive selection for rapid growth and meiosis in the diploid host while imposing little negative selection pressure for high infectivity of the virus. It also leads to the prediction that *E. huxleyi N* cells do not simply act as gametes by mating at the first opportunity, as this would produce 2*N* cells susceptible to viral attack in an environment where viruses are still present in high concentrations. Rather, the noncalcifying haploid phase probably plays a prominent ecological role, dividing and migrating with the consequence of temporally and spatially displacing an eventual inoculum of novel 2*N* cells.

Evolutionary Significance: Red Queen or Cheshire Cat?

Originally proposed by Van Valen (Van Valen 1973), the metaphor of an evolutionary ‘‘arms race’’ has been widely used for describing various biotic interactions and termed Red Queen (RQ) dynamics in reference to the Red Queen’s race in Lewis Carroll’s *Through the Looking Glass*, in which the Red Queen states ‘‘it takes all of the running you can do, to keep in the same place’’ (Carroll 1865). The idea that genetic recombination through sex could be key in the evolutionary arms race between parasites and hosts was developed later by several authors (e.g., refs. (Jaenicke 1978, Bell 1994)). Our results indicate that viral infection of the diploid stage of *E. huxleyi* promotes sexual cycling, either directly by inducing meiosis and/or indirectly by removing the more competitive 2*N* cells. A classical RQ interpretation would be that this sexual cycling leads to increased diversity in the following diploid generation, and that among this diversity certain genotypes would be more resistant to viral attack, and therefore positively selected. The fact that EhVs typically infect a limited range of diploid *E. huxleyi* strains in culture (Schroeder et al. 2002) may support this interpretation. However, the limited data available from natural populations do not indicate a constant and rapid turnover of coevolving *E. huxleyi* and EhV genotypes in the oceans. In our experiments, an EhV strain isolated from the North Atlantic was capable of infecting host strains originating from the Mediterranean Sea (RCC1249, RCC1213) and from the Tasman Sea off New Zealand (RCC1216). Rapid RQ dynamics would presumably have led to localized, genetically distinct subpopulations of host and virus, at least in terms of resistance and recognition genes,

respectively. Furthermore, the *GPA* gene, encoding a highly polymorphic protein with calcium-binding motifs and used as a genotype marker in *E. huxleyi*, did not reveal variation of the dominant genotypes over multiannual mesocosm experiments in Norwegian fjords (Martinez et al. 2007), suggesting the existence of an efficient strategy for survival of these genotypes between blooms. The persistence of *E. huxleyi* strains over multiple years in the North Atlantic, the lack of biogeographic structuring in *E. huxleyi*–EhV infectivity patterns, and in general the maintenance of elevated growth rate and infectivity in *E. huxleyi* and EhV, respectively, argue against the existence of a classical, highly dynamic RQ equilibrium in this host–virus system. The invisibility of the host haploid stage to the virus circumvents, or at least drastically slows, the arms race. The period of respite experienced during one life cycle phase means that resources of the phase susceptible to viral attack can be focused on interactions with direct ecological competitors rather than on developing new arms against the virus. In keeping with the RQ metaphor taken from Lewis Carroll, we liken this theory to the strategy used by the Cheshire Cat in *Alice's Adventures in Wonderland* (Carroll 1865) of making its body invisible to make the sentence ''off with his head'' pronounced by the Queen of Hearts impossible to execute. RQ and Cheshire Cat (CC) mechanisms, both of which act around a central eukaryotic process, sexual reproduction, should not be considered mutually exclusive. CC dynamics, which rely to some extent on separation of the sexual processes of meiosis and fusion in time and/or space, release the host from short-term pathogen pressure, thus widening the scope for the host to evolve in other directions. Evolution of genes conferring resistance to viral attack in the host and the counteractive evolution of new arms by the virus likely still occur, but over much longer time scales than classically inferred. *E. huxleyi* is a relatively young species (or species complex), having evolved from the Gephyrocapsids some 270,000 years ago (Thierstein et al. 1977), and thus represents an interesting model for assessment of the pace of host–virus coevolution and the interaction between the Cat and the Queen. CC dynamics could, of course, also apply to predator–prey interactions. Cases of phenotypic changes linked to predation pressure have been documented in protists (Boraas et al. 1998); however, to our knowledge there are, as yet, no reports of phenotypic switches associated with ploidy changes under these conditions.

4. Concluding Remarks

Each milliliter of seawater is known to contain millions of viral particles, and the growing evidence of an enormous diversity of eukaryotic viruses (Suttle 2007) and parasites (Kim et al. 2008) suggests that each eukaryote species, and potentially each clonal strain in

the oceans, has its own pathogen(s). Our data reveal a fundamental mechanism that may correlate the massive diversity of oceanic pathogens and the maintenance of dimorphic sexual life cycles in marine eukaryotes. Understanding these relationships involves more than mere intellectual curiosity on the coevolution of sex and viruses. In fact, eukaryotic marine protists are responsible for nearly half of global primary productivity and control most of the flux of matter between the atmosphere and the lithosphere (Falkowski et al. 2004). The fate of oceanic primary production, whether sedimented to the ocean floor or remineralized in surface waters, depends fundamentally on the physical properties of the eukaryotic cells themselves, which change dramatically as they differentiate over their life cycle. In the case of *E. huxleyi*, the impact of each life cycle phase on Earth System dynamics is obviously radically different: the diploid stage is responsible for a significant amount of planetary carbonate production and is thus heavily implicated in global climate regulation (Tyrrell & Merico 2004), whereas the haploid stage does not calcify and is probably mostly remineralized in surface waters. Understanding the genomic controls of pathogen resistance and ploidyrelated phenotypic differentiation in oceanic protists will significantly advance assessment and prediction of their impact on biogeochemical cycles.

4. Methods

Strains and Culture Conditions

The *E. huxleyi* strains used in this study, RCC1216 (origin: Tasman Sea, New Zealand), RCC1249 (origin: Mediterranean Sea, Spain), and RCC1213 (origin: Mediterranean Sea, Italy) from the Roscoff Culture Collection, France (http://www.sb-roscoff.fr/Phyto/RCC), were originally initiated by micropipette isolation of a single diploid (coccolith-bearing) cell. After diploid-to-haploid life cycle transitions of a few cells in the original cultures, pure cultures of each phase were established from the mixed-phase cultures by single-cell micropipette isolation. Culture purity was verified by light microscopy before each experiment. Experiments were conducted in triplicate in K/2 (minus Si, minus Tris) medium at 18°C, 12:12 (light:dark) cycle and 85 $\mu E.m^{-2}.s^{-1}$ irradiance. The viral strains (EhV84, EhV86, EhV88, EhV163, EhV201, EhV202, EhV203, EhV204, EhV205, EhV206, EhV207, EhV208, EhV209, EhVv1, and EhVv2; ref. (Bratbak et al. 1993)) were maintained at 4°C in K/2 medium and filtered through a 0.2 µm filter (Minisart; Sartorius) before utilization.

Flow Cytometry and Photosynthetic Activity Analyses

Enumeration of *E. huxleyi* cells and viruses was performed with a FACSCalibur flow cytometer (Becton Dickinson) equipped with an air-cooled laser providing 15mW at 488 nm and with the standard filter setup. Haploid and diploid *E. huxleyi* cells in unfixed samples were identified on the basis of their chlorophyll fluorescence and side-scatter signatures (Fig. S4). Virus enumeration was performed according to Marie *et al.* (Marie et al. 1999).A FACS Aria flowcytometer (Becton Dickinson) was used for sorting *E. huxleyi* haploid and diploid cells. The photosynthetic activity of cells was quantified by measuring variable fluorescence before and during a saturating light pulse (0.6 s, 470 nm, 1,700 $\mu mol.m^{-2}.s^{-1}$) with a PHYTO-PAM (Mess und Regaltechnik) using fresh samples incubated for 10 min in the dark before analyses. The quantum efficiency of photosystem II (Fv/Fm) was calculated by $Fv/Fm = (Fm - F0)/Fm$, where $F0$ is the minimum fluorescence and Fm is the maximum fluorescence of the dark-adapted sample during the saturating light pulse.

Infection Assays

The first experiment was designed to assess the response of the haploid and diploid phases of all *E. huxleyi* strains to infection with each of the viral strains. Exponentially growing cultures (200 ml; ~2 x 10^6 cells per ml) of *E. huxleyi* strains were infected independently with each virus strain at a virus-to-cell ratio (MOI) of 0.2. Enumeration of algae and viruses and measurement of photosynthetic activity were performed daily. Controls without addition of viruses were performed in parallel. The *E. huxleyi* RCC1216 *N* and 2*N* strains and the EhV201 viral strain were chosen for further experiments. The effect of addition of various viral concentrations on the haploid phase of *E. huxleyi* was then tested. Cultures of the haploid stage used in the previous infection experiment (including controls) were diluted 200 times in 200 ml of fresh K/2 (minus Si, minus Tris) medium and incubated in conditions previously described. After attaining ~2 x 10^6 cells per ml, duplicates were inoculated with mois of 0.05, 1, and a control (no virus addition). Control cultures, which had never been in contact with viruses, were inoculated with MOIs of 0.5, 5, and a control. Samples for enumeration of algae and viruses were collected six and three times, respectively, at irregular intervals over a 26-day period.

Long-Term Infection Assays

To mimic a bloom situation based on the observations of Castberg *et al.* (Castberg et al. 2001) and Jacquet *et al.* (Jacquet et al. 2002), 200-ml exponentially growing diploid cultures were inoculated with haploid cells in exponential growth at a $2N/N$ cell ratio of 100

(~1 x 10^6 2*N* cells per ml and 1 x 10^4 *N* cells per ml), and infected with viruses at an MOI of 0.2. Noninfected diploid cultures with and without addition of haploid cells were used as controls. Populations of *N* and 2*N* cells of *E. huxleyi* and viruses were monitored for 50 days. In the initial stages of infection, cells from the samples were sorted by flow cytometry and cultured in 2 ml of K/2 (minus Si, minus Tris) medium.

DNA Extraction and PCR Amplification

To assess whether viruses were sticking to and/or penetrating *E. huxleyi* haploid cells,weattempted to PCR amplify the viral major capsid protein (MCP) gene (~300 kb; ref. (Schroeder et al. 2002)) from infected haploid and diploid cells after multiple washes. Samples from *N* and 2*N* cultures were collected 2 days after viral addition. Free-floating viral particles were washed away with fresh medium by multiple filtrations of the cells on polyethersulfone membranes (0.45-µm pore size, GE Osmonics Labstore). TotalDNAwas then extracted from the cells on the filters (Shaked & De Vargas 2006). PCR amplifications with the primer pair MCP-F and MCP-R were performed according to Schroeder *et al.* (Schroeder et al. 2002). A pure extract of EhV201 DNA was used as a positive control.

Transmission Electronic Microscopy

For thin sectioning, the cells were fixed for 1.5 h in 4% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2) with 0.25 M sucrose. Cells were then washed three times in 0.1 M sodium cacodylate containing decreasing concentrations of sucrose and were postfixed in 2% osmium tetroxide in 0.1 M sodium cacodylate for 1.5 h. After washing in distilled water, cells were dehydrated in a graded ethanol series and embedded in Epon resin. Sections were double stained with uranyl acetate followed by Reynold lead citrate. Observations were carried out on a JEOL JEM 1011 electron microscope.

5. Acknowledgements

We thank the Plancton Océanique group at the Station Biologique de Roscoff, especially D. Marie, F. Le Gall, A. Pagarete, P. Von Dassow, and E. M. Bendif, for assistance with flow cytometry and algal cultures, and for informative discussions.Wealso thank J. Barcelos e Ramos for sharing observations. This work was supported by a Ph.D. fellowship awarded to M.F. by the Fundação Para a Ciência e para a Tecnologia, Portugal, and an Actions Thématiques et Incitatives sur Programme (ATIP) grant awarded to C.d.V. by the Centre National de la Recherche Scientifique, France. It is part of the pluridisciplinary project

BOOM (Biodiversity of Open Ocean Microcalcifiers) funded by the French Agence Nationale de la Recherche, Grant ANR-05-BIODIV-004.

6. References

Allen MJ, Schroeder DC, Holden MTG, Wilson WH (2005) Evolutionary history of the Coccolithoviridae. Molecular Biology and Evolution

Ameisen JC (2002) On the origin, evolution, and nature of programmed cell death: a timeline of four billion years. Cell Death and Differentiation 9:367-393

Baranowski E, Ruiz-Jarabo CM, Domingo E (2001) Evolution of Cell Recognition by Viruses. Science 292:1102-1105

Bell G (1994) The comparative biology of the alternation of generations. In: Kirpatrick M (ed) Lectures on Mathematics in Life Sciences: The Evolution of Haplo-Diploid Life Cycles. American Mathematical Society, Rhode Island, p 1-26

Bidle KD, Haramaty L, Barcelos e Ramos J, Falkowski P (2007) Viral activation and recruitment of metacaspases in the unicellular coccolithophore, Emiliania huxleyi. PNAS 104:6049-6054

Billard C (1994) Life cycles. In: Green JC, Leadbeater BSC (eds) The Haptophyta Algae, Vol 51. Clarendon Press, Oxford, p 167-186

Bishop NE, Anderson DA (1997) Early interactions of hepatitis A virus with cultured cells: viral elution and the effect of pH and calcium ions. Archives of Virology 142:2161-2178

Bohannan BJM, Lenski RE (2000) Linking genetic change to community evolution: insights from studies of bacteria and bacteriophage. Ecol Letters 3:362-377

Boraas ME, Seale DB, Boxhorn JE (1998) Phagotrophy by a flagellate selects for colonial prey: a possible origin of multicellularity. Evolutionary Ecology 12:153-164

Bratbak G, Egge J, Heldal M (1993) Viral mortality of the marine alga *Emiliania huxelyi (*Haptophyceae) and the termination of the algal bloom. Marine Ecology Progess Series 93:39-48

Brownlee C, Taylor AR (2004) Calcification in coccolithophores. In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Brussaard CPD (2004) Viral Control of Phytoplankton Populations - a Review. The Journal of Eukaryotic Microbiology 51:125-138

Brussaard CPD, Kuipers B, Veldhuis MJW (2005) A Mesocosm Study of *Phaeocystis globosa* population dynamics I. Regulatory Role of Viruses in Bloom Control. Harmful Algae 4:859-874

Carroll L (1865) Alice's adventures in wonderworld Vol. Signet Classics, New York, London

Castberg T, Larsen A, Sandaa RA, Brussard CPD, Egge JK, Heldal M, Thyrhaug R, van Hannen EJ, Bratbak G (2001) Microbial population dynamics and diversity during a bloom of the marine coccolithophorid Emiliania huxleyi (Haptophyta). Marine Ecology Progress Series 221:39-46

de Vargas C, Aubry M-P, Probert I, Young J (2007) Origin and evolution of coccolithophores: from coastal hunters to oceanic farmers. In: Falkowski P, Knoll AH (eds) Evolution of Aquatic Photoautotrophs. Elsevier Academic Press, New York, p 251-285

Evans C, Malin G, Mills GP, Wilson WH (2006a) Viral Infection of *Emiliania huxleyi* (Prymnesiophyceae) Leads to Elevated Production of Reactive Oxygen species. Journal of Phycology 42:1040-1047

Evans C, Malin G, Wilson W, Liss P (2006b) Infectious titres of *Emiliania huxleyi* virus 86 are reduced by exposure to millimolar dimethyl sulfide and acrylic acid. Limnol Oceanogr 51:2468–2471

Falkowski PG, Katz ME, Knoll AH, Quigg A, Raven JA, Schofield O, Taylor FJR (2004) The Evolution of Modern Eukaryotic Phytoplankton. Science 305:354-360

Green JC, Course PA, Tarran GA (1996) The life-cycle of Emiliania huxleyi: A brief review and a study of relative ploidy levels analysed by flow cytometry. Journal of Marine Systems 9:33-44

Houdan A, Billard C, Marie D, Not F, Saez A, Young G, Probert I (2004) Holococcolithophores-heterococcolithophores (Haptophyta) life cycles: flow cytometry analysis of relative ploidy levels. Systematics and Biodiversity 1:453-465

Houdan A, Probert I, Van Lenning K, Lefebvre S (2005) Comparison of photosynthetic responses in diploid and haploid life-cycle phases of *Emiliania huxleyi* (Prymnesiophyceae). Marine Ecology Progress Series

Houdan A, Probert I, Zatylny C, Véron B, Billard C (2006) Ecology of oceanic coccolithophores. I. nutritional preferences of the two stages in the life cycle of *Coccolithus braarudii* and *Calcidiscus leptoporus*. Aquatic Microbial Ecology 44:291-301

Huheihel M, Ishanu V, Tal J, Arad S (2002) Activity of Porphyridium sp. polysaccharide against herpes simplex viruses in vitro and in vivo. J Biochem Biophys Methods 50:189-200

Iglesias-Rodriguez MD, Halloran PR, Rickaby REM, Hall IR, Colmenero-Hidalgo E, Gittins JR, Green DRH, Tyrrell T, Gibbs SJ, von Dassow P, Rehm E, Armbrust EV, Boessenkool KP (2008) Phytoplankton calcification in a high-CO_2 world. Science 320:336-340

Jacobsen A, Bratbak G, Heldal M (1996) Isolation and characterization of a virus infecting *Phaeocystis pouchetii* (Prymnesiophyceae). Journal of Phycology 32:923-927

Jacquet S, Heldal M, Iglesias-Rodriguez D, Larsen A, Wilson W, Bratbak G (2002) Flow cytometric analysis of an Emiliania huxleyi bloom terminated by viral infection. Aquatic microbial ecology 27:111-124

Jaenicke J (1978) An hypothesis to account for the maintenance of sex within populations. Evol Theory 3:191–194

Kim S, Park MG, Kim K-Y, Kim C-H, Yih W, Park JS, Coats DW (2008) Genetic Diversity of Parasitic Dinoflagellates in the Genus Amoebophrya and Its Relationship to Parasite Biology and Biogeography. The Journal of Eukaryotic Microbiology 55:1-8

Marie D, Brussaard CPD, Thyrhaug R, Bratbak G, Vaulot D (1999) Enumeration of marine viruses in culture and natural samples by flow cytometry. Appl. Environ. Microbiol. 65:45-52

Martinez JM, Schroeder DC, Larsen A, Bratbak G, Wilson WH (2007) Molecular Dynamics of Emiliania huxleyi and Cooccurring Viruses during Two Separate Mesocosm Studies. Appl. Environ. Microbiol. 73:554-562

Nedelcu AM, Michod RE (2003) Sex as a response to oxidative stress: the effect of antioxidants on sexual induction in a facultatively sexual lineage. Proc. R. Soc. Lond. Biological Sciences 270:136 -139

Noel M-H, Kawachi M, Inouye I (2004) Induced dimorphic life cycle of a coccolithophorid, *Calyptrosphaera sphaeroidea* (Prymnesiophyceae, Haptophyta). Journal of Phycology 40:112-129

Paasche E (2001) A review of the coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae), with particular reference to growth, coccolith formation, and calcificationphotosynthesis interactions. Phycologia 40:503-529

Riebesell U, Zondervan I, Rost B, Tortell PD, Zeebe RE, Morel FMM (2000) Reduced calcification of marine plankton in response to increased atmospheric CO2. Nature 407:364-367

Schroeder D, Oke J, Malin G, Wilson WH (2002) Coccolithovirus (Phycodnaviridae): Characterisation of a new large dsDNA virus that infects *Emiliania huxleyi*. Archives of Virology 147:1685-1698

Shaked Y, De Vargas C (2006) Pelagic photosymbiosis: rDNA assessment of diversity and evolution of dinoflagellate symbionts and planktonic foraminiferal hosts. Marine Ecology Progess Series 325:59-71

Suttle CA (2007) Marine viruses - major players in the global ecosystem. Nat Rev Micro 5:801-812

Tarutani K, Nagasaki K, Yamaguchi M (2000) Viral impacts on total abundance and clonal composition of the harmful bloom-forming phytoplankton *Heterosigma akashiwo*. Appl. Environ. Microbiol. 66:4916-4920

Thierstein H, Geitzenauer K, Molfino B, Shakleton N (1977) Global synchroneity of late Quarternary coccolith datum levels: Validation by oxygen isotopes. Geology 5:400-4004

Thingstad TF (2000) Elements of a theory for the mechanisms controlling abundance, diversity, and biogeochemical role of lytic bacterial viruses in aquatic systems Limnol Oceanogr 45:1320–1328

Thornber CS (2006) Functional Properties of the Isomorphic Biphasic Algal Life Cycle. Integrative and Comparative Biology 46:605-614

Tyrrell T, Merico A (2004) *Emiliania huxleyi*: bloom observation and the conditions that induce them. In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Valero M, Richerd S, Perrot V (1992) Evolution of alternation of haploid and diploid phases in life cycles. Trends in Ecology and Evolution 7:25-29

Van Valen L (1973) A new evolutionary law. Evolutionary Theory 1:1-30.

Vardi A, Berman-Frank I, Rozenberg T, Hadas O, Kaplan A, Levine A (1999) Programmed cell death of the dinoflagellate Peridinium gatunense is mediated by CO2 limitation and oxidative stress. Current Biology 9:1061-1064

Wilson WH, Schroeder D, Allen M, Holden MTG, Parkhill J, Barrell BG, Churcher C, Hamlin N, Mungall K, Norbertczak H, Quail MA, Price C, Rabbinowitsch E, Walker D, Craigon M, Roy D, Ghazal P (2005) Complete Genome Sequence and Lytic Phase Transcription Profile of a Coccolithovirus. Science 309

Wilson WH, Tarran GA, Schroeder D, Cox M, Oke J, Malin G (2002) Isolation of viruses responsible for the demise of an *Emiliania huxleyi* bloom in the English Channel. J. Mar. Biol. Ass. U.K. 82:369-377

7. Supporting Information

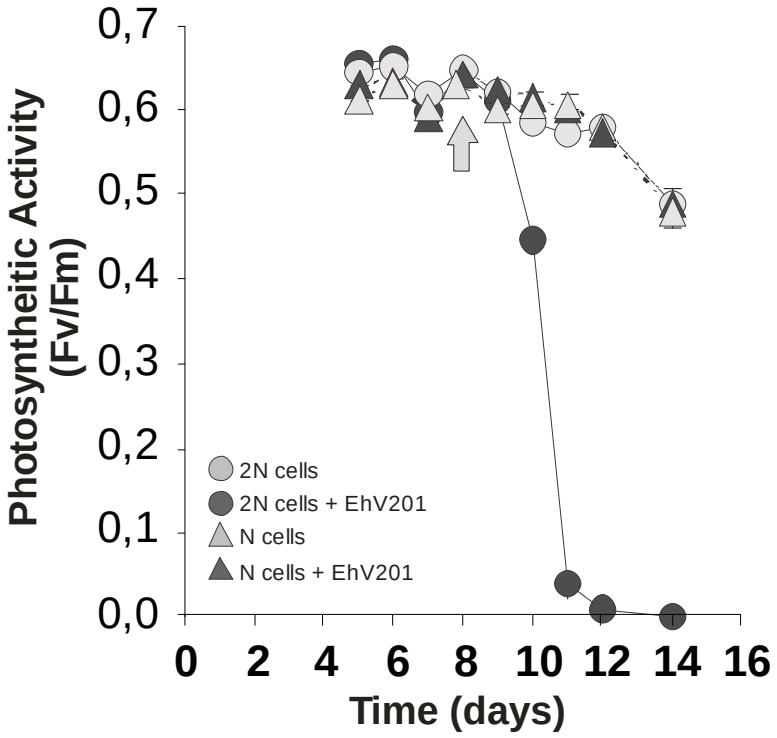


Fig. S1. Photosynthetic activity of both life cycle stages of *Emiliania huxleyi* (RCC1216) with and without virus addition. *F*v/*F*m, quantum efficiency of photosystem II. The arrow indicates the day of virus addition. Standard deviation bars are generally too short to be visible.

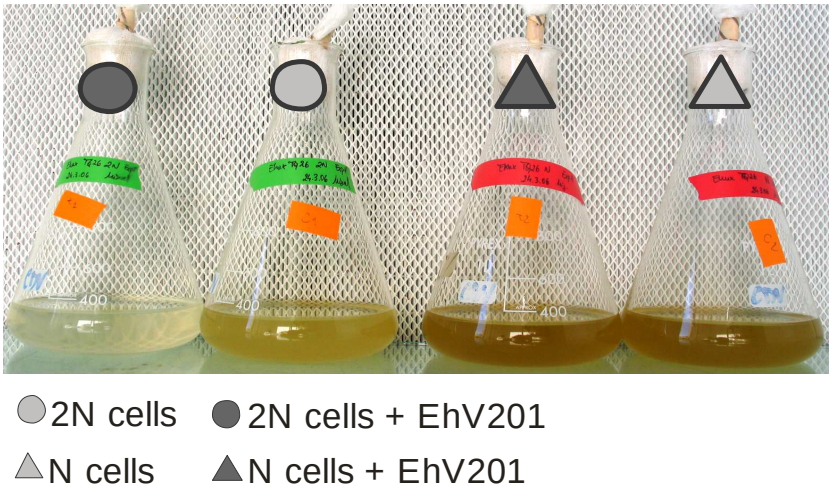


Fig. S2. Series of culture flasks at day 11 (the 11-day time point in Fig. 14).

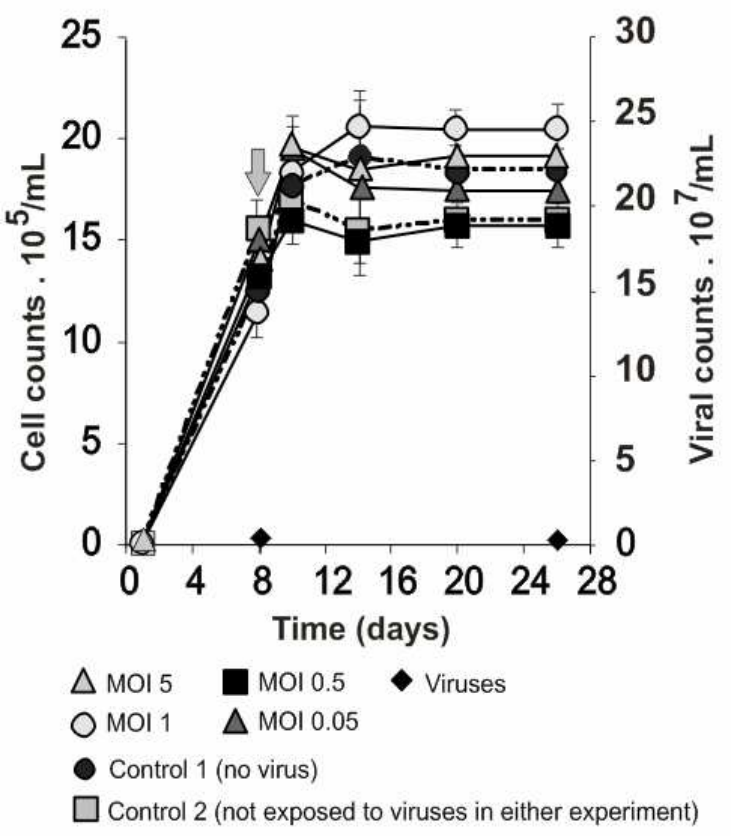


Fig. S3. Reinfection assays of *E. huxleyi N* cultures at various viral concentrations. The *N* cultures used in the experiment shown in Fig. 16 were diluted 200-fold, grown up to~2 x 10^6 cells per ml, and subjected to anewvirus inoculation at various MOIs. The arrow indicates the time of virus addition. The error bars represent the range between duplicate assays.

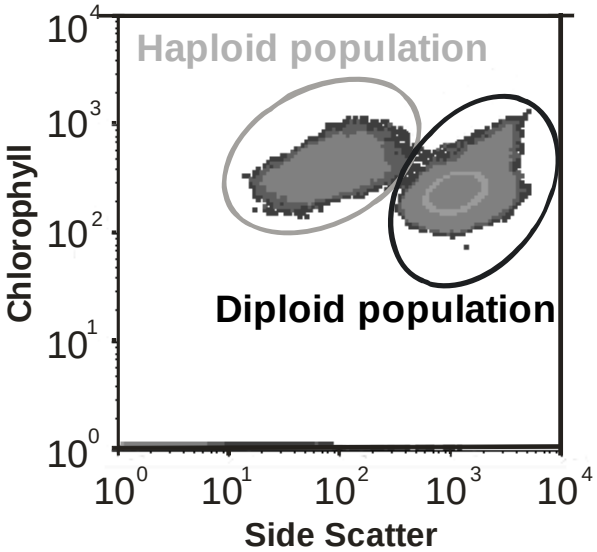


Fig. S4. Flow cytometric plot of *E. huxleyi* (RCC1216) noncalcified *N* and calcified 2*N* life cycle stages. The 2*N* cells can lose their coccoliths and consequently fall in the right part of the *N* window. This potential problem in quantification of life cycle stages was circumvented by the use of controls and light microscopy observations of motility (*N* cells are flagellated). Only cells falling inside the flow cytometer windows corresponding to the *N* and 2*N* populations were taken into account during the experiments.

CHAPTER 2. $CaCO_3$ optical detection with fluorescent *in situ* hybridization: a new method to identify and quantify calcifying microorganisms from the oceans

$CaCO_3$ optical detection with fluorescent *in situ* hybridization: a new method to identify and quantify calcifying microorganisms from the oceans

Miguel Frada[1,2]*, Fabrice Not[1], Ian Probert[3], Colomban de Vargas[1]

[1]CNRS & Université Pierre et Marie Curie (UMR 7144), Equipe EPPO - Evolution du Plancton et Paléo-Océans, Station Biologique, 29682 Roscoff, France

[2]Departamento de Geologia, Faculdade de Ciências, Universidade de Lisboa, Edifício C6, Campo Grande, 1749-016 Lisboa, Portugal

[3]Algobank-Caen Culture Collection, Université de Caen, 14032 Caen, France

*corresponding author:

Miguel Frada

Station Biologique

Equipe EPPO - Evolution du Plancton et Paléo-Océans (UMR 7144)

Place Georges Teissier, Roscoff (France)

Phone : 33 (0) 2-98-29-25-28

email : frada@sb-roscoff.fr

Published in : **Journal of Phycology, 2008 vol. 42 1162-1169**

- Attached at the end of the manuscript there is a detailed protocol of the COD-FISH method that was published online together with the publication (**Annex 2**)

⌬ the structure of the manuscript submitted to the Journal of Phycology were maintained

Abstract

Open oceanic calcification is mainly driven by unicellular organisms and in particular by eukaryotes such as coccolithophores and foraminifers. Open ocean microcalcifiers, like most planktonic protists, are characterized by extremely fast generation times and occasional sexual reproduction. Populations can alternate between diploid and haploid stages, which often build different kinds of cell covers. In the most important pelagic calcifiers, the coccolithophores, the diploid and haploid stages, which can self-replicate and grow independently, display radically different morphologies with different modes of calcification or even with the absence of calcification in at least one life cycle stage. Although life cycle strategies seem likely to fundamentally influence the where and when of open ocean calcification, this issue has yet to be seriously addressed in the natural environment. Here, we introduce a new morphogenetic method, ‘‘combined CaCO3 optical detection with fluorescent in situ hybridization,’’ or COD-FISH, which is based on a combination of TSA-FISH and polarized optical microscopy. This technique allows simultaneous assessment of the taxonomic and life cycle status of single coccolithophore cells collected from the ocean. We demonstrate the application of CODFISH using both laboratory culture and field samples and discuss its potential value for assessing the ecology, biodiversity, population structure, and life cycles of coccolithophores and other open ocean unicellular calcifiers.

Key index words

calcareous plankton; calcification; coccolithophore; COD-FISH; haplo-diploidy; heteromorphy; life cycle; open ocean

Abbreviations

COD-FISH, combined $CaCO_3$ optical detection with fluorescent in situ hybridization; CPM, cross-polarized microscopy; HRP, horseradish peroxidase; TSA, tyramide signal amplification

1. Introduction

The coccolithophores (Prymnesiophyceae, Haptophyta) are unicellular marine phytoplankton with worldwide distributions, defined by the presence of extracellular calcified scales, the coccoliths, at some stage of their life cycles. They are ecologically highly significant, both as primary producers and for certain species (e.g. *Emiliania huxleyi*) as bloom-forming organisms. The processes of photosynthesis, respiration, and calcification occurring in large coccolithophore blooms have substantial implications in the global cycles of carbon and sulfur (Rost et al. 2003, Malin & Steinke 2004, Tyrrell & Merico 2004). After cell death, the coccoliths can be transported to the seafloor, where they have formed over the last 200 million years one of the most commonly used fossil records for stratigraphic and paleoceanographic studies.

A classical, simple, and efficient way to observe and study coccolithophores is by cross-polarized microscopy (CPM). This technique enables assessment of the crystallographic orientation of the calcareous coccoliths, which is taxon (morphospecies) and life cycle stage specific (Young et al. 1992, Young et al. 2003). Using CPM and various other techniques in microscopy, the extant diversity of coccolithophores has been divided into about 280 morphological species (Young et al. 2003). However, this number is undoubtedly biased by two opposing phenomena (de Vargas & Probert 2004). Firstly, the coccolithophores display complex but poorly known haplo-diploid life cycles (Billard 1994, Houdan et al. 2004). Current evidence suggests that diploid cells are typically covered with heterococcoliths that are produced intracellularly and are formed of complex interlocking crystal units. In their haploid stage, the cells express a radically different phenotype, ranging from the production of holococcoliths, formed by numerous, minute discrete crystals that seem to be precipitated extracellularly, to the total absence of biomineralization (Fig. 17). The ~60 recognized holococcolith- bearing coccolithophores most likely represent haploid stages of diploid heterococcolith-bearing species. This phenomenon will thus decrease current estimates of coccolithophore biodiversity. Inversely, the recent application of molecular tools suggests that the diversity of coccolithophore speciesmay be significantly higher than currently believed (Moon-Van der Staay et al. 2000). As seems to be the case in diverse groups of marine planktonic organisms, the classical morphological species typically consist of monophyletic groups of sister, cryptic or pseudocryptic, biological species (Saez et al. 2003, de Vargas et al. 2004). Thus, while classical morphological observations appear less powerful than molecular methods as taxonomic tools, the DNA data totally ignore fundamental biological features of the organisms, such as the life cycle stage or the calcification state of the cell. Morphogenetic

protocols that allow simultaneous analysis of both morphological and molecular identities are required to improve our knowledge of coccolithophore biodiversity and ecology.

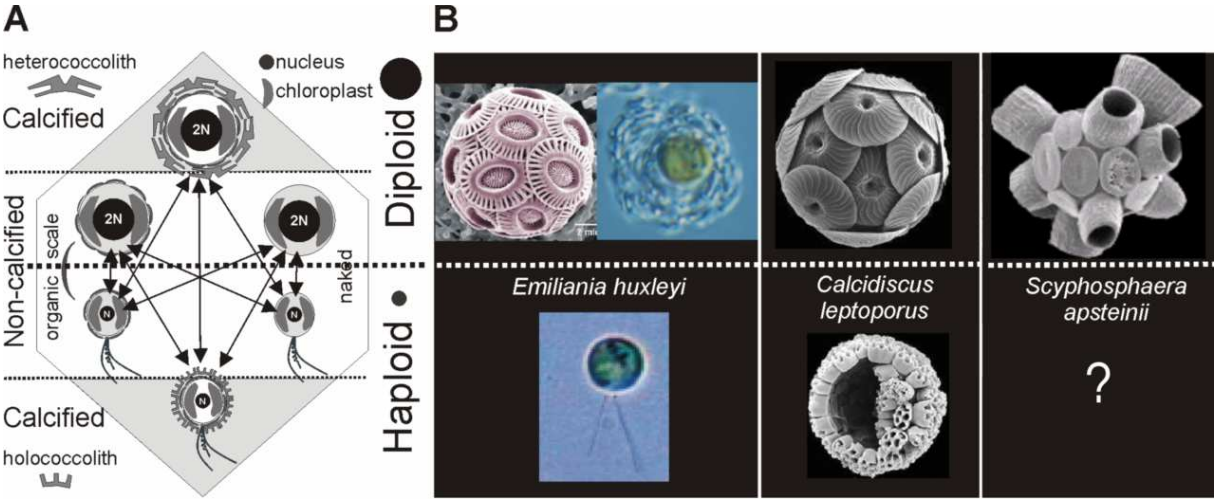


Figure 17. Coccolithophore life cycles and morphological diversity (modified from de Vargas and Probert 2004). (A) The hexagon of lifecycle in coccolithophores. In their diploid and haploid life-cycle stages, the coccolithophores may potentially be calcified, covered with organic scales, or simply naked. Theoretically, all of the nine life-cycle associations shown here (arrows) are possible, which makes estimation of coccolithophore diversity using simple morphological analyses of the coccoliths unfeasible. (B) Morphological diversity among and within the coccolithophore species used in this study. *Emiliania huxleyi*: diploid stage with multiple layers of heterococcoliths (top)/haploid noncalcifying, organic scale-bearing cell (bottom); *Calcidiscus leptoporus*: diploid stage with a single layer of heterococcoliths (top)/haploid cell with a single layer of holococcoliths (bottom); *Scyphosphaera apsteinii*: diploid stage with a single layer of heteromorphic heterococcoliths (top). The haploid stage of *S. apsteinii* is presently unknown (bottom).

Whole cell FISH with rRNA-targeted oligonucleotide probes has been widely used to identify free-living bacteria and protists (Amann et al. 1995, Simon et al. 2000, Amann et al. 2001). The application of the classical FISH-protocol using monolabeled probes to populations of eukaryotic phytoplankton was initially limited by the natural autofluorescence of the pigmented cells, which interferes significantly with the fluorescent signal from the probes. This problem of sensitivity was overcome with the advent of fluorescence amplification systems. Schonhuber et al. (Schönhuber et al. 1997) introduced TSA-FISH, a method using fluorescent tyramides as the substrates for probes labeled with a horseradish peroxidase (HRP) enzyme. This approach, which increases the signal 10–20 times over that of monolabeled probes, has recently proved to be a powerful technique for the identification and quantification of phytoplankton cells in cultures and natural samples (Biegala et al. 2002, Not et al. 2002). The main remaining problem of FISH sensu lato, and especially for application

of the method to larger eukaryotic cells displaying complex phenotypes, is to link the genetic signature to the morphology of the cell. In this context, Stoeck et al. (Stoeck et al. 2003) demonstrated the feasibility of associating FISH detection with SEM, for morpho-molecular characterization of single heterotrophic protist cells. The protocol for combined $CaCO_3$ optical detection with fluorescent in situ hybridization, or COD-FISH, presented here explores similar concepts for application in unicellular calcifying organisms. Major modification of the TSA-FISH protocol allowed us to genetically identify single coccolithophore cells while preserving both the CaCO3 crystals of their coccoliths and the full geometric structure of their complex skeleton, or coccosphere, built of multiple coccoliths (Fig. 17). The three main goals of this work were to develop a protocol that (1) allows combined morpho-molecular examinations of individual unicellular calcareous organisms, (2) is a quantitative method and permits accurate enumeration of calcifying versus noncalcifying cells, and (3) can be applied to natural marine microbial communities.

2. Material and methods

Culture samples and estimation of cell densities. The coccolithophore species used in this study belong to three orders within the Prymnesiophyceae: Isochrysidales, Coccolithales, and Zygodiscales. Three additional microalgal strains belonging to the Prasinophyceae, Chlorophyceae, and Pelagophyceae (Table 2) were used as negative controls to test the specificity of rDNA probes. Cultures were maintained in both haploid and diploid stages in Nalgene Nunc flasks (Rochester, NY, USA), in K (-Tris, -Si) medium (Keller et al. 1987) at a temperature of 20º C, except for the Isochrysidales which were grown at 18º C. While testing the COD-FISH protocol, various mixes of the different cultures were artificially created. For each strain, cell densities were first estimated by flow cytometry on live samples before mixing (Marie et al. 1999). After the fixation and filtration steps of the hybridization protocol, 4',6-diamidino-2-phenylindole (DAPI, Sigma Inc., St. Louis, MO, USA) stained cells (on membrane filters) were counted using an epifluorescence microscope for a reassessment of cell densities. The same membranes were then used to perform COD-FISH, and counts were ultimately compared. To test the accuracy of COD-FISH as a quantitative method, we mixed pure haploid and diploid cultures of *E. huxleyi*. After filtration onto membranes, the number of diploid heterococcolith bearing cells was estimated by microscope, counting under cross-polarized visible light, while the number of naked haploid cells was calculated by subtracting the number of diploid cells from the total number of cells counted

under fluorescence (DAPI or fluorecein isothiocyanate [FITC], Perkin Elmer, Wellesley, MA, USA).

Table 2. Algal strains used in this study.

Strain Identification number	Division	Order	Genus & species	Life cycle stage
AC472	Haptophyta	Isochrysidales	*Emiliania huxleyi*	Diploid, heterococcolith-bearing
AC472	Haptophyta	Isochrysidales	*Emiliania huxleyi*	Haploid non-calcifing
AC370	Haptophyta	Coccolithales	*Calcidiscus leptoporus*	Diploid, heterococcolith-bearing
AC370	Haptophyta	Coccolithales	*Calcidiscus leptoporus*	Haploid, holococcoliths-bearing
AC505	Haptophyta	Zygodicales	*Scyphosphaera apsteini*	Heterococcoliths-bearing cells, presumably diploid
RCC114	Chlorophyta	**Mamiellales**	*Micromonas sp.*	-
RCC1	Chlorophyta	Chlamydomonadales	*Chlamydomonas concordia*	-
RCC101	Heterokontophyta	Pelagomonadales	*Pelagomonas calceolata*	-

AC, Algobank Culture Collection; RCC, Roscoff Culture Collection.

Environmental samples. Environmental samples were collected on two occasions. Firstly, 2.5 L of surface seawater was collected on December 14, 2004, from the pier in front of the ‘‘Station Biologique’’ (Roscoff, France) using a plastic bucket. This sample was prefiltered through a 20 mm nylon mesh to remove larger organisms and particles and concentrated 100 times by tangential flow filtration using a VIVA FLOW 200 filtration unit (Viva Science, LDA, UK). The second set of samples was collected during a mesocosm experiment in May/June 2005 at the Marine Biological Field Station of Espeland (Bergen, Norway). Nine $11m^3$ polyethylene bags were filled with surrounding seawater, and a bloom of the coccolithophore species *E. huxleyi* was artificially induced as previously described (Delille et al. 2005). For the present study we used samples from three mesocosms (numbers 2, 5, and 8) collected during the exponential phase of the bloom development (May 23). Cell densities, for total picoeukaryotes, nanoflagellates, and *E. huxleyi* were estimated by flow cytometry on live samples based on their fluorescence and light scatter.

The COD-FISH protocol. The COD-FISH method is based on TSA-FISH protocols previously developed for photosynthetic picoeukaryotes (Not et al. 2002). The genetic detection of the coccolithophore species was achieved using the Haptophyta-specific probe PRYM02 (50-GGA ATA CGA GTG CCC CTG AC-30) labeled with an HRP enzyme (Simon et al. 2000). Here we detail only the crucial modifications made to each step of the TSA-FISH protocol (Not et al., 2002) that led to COD-FISH (a full version of the protocol can be downloaded at http://www.blackwell-synergy.com) (1) Fixation. 6mM Na_2CO_3 (final concentration) was added to the paraformaldehyde fixative (PFA) to prevent any dissolution of the calcareous coccoliths. (2) Filtration. After low vacuum filtration of the PFA-fixed seawater and an ethanol dehydration series, the 25mm Anodisc filters (Whatman, Maidstone, Kent, UK) were covered with 1mL of prewarmed, 1% liquid gelatin (dissolved in 6mM Na_2CO_3) for 3min. After polymerization, the gelatin ensured the maintenance of the integrity of the composite coccospheres and prevented cell losses from the Anodisc membranes. The filters were then dried for 1 h at 30º C and stored in Petrislides (Millipore, Bedford, MA, USA) at - 80º C until further processing. (3) Hybridization. Once again to avoid the dissolution of calcareous microskeletons, 6mM Na_2CO_3 (final concentration) was added to the hybridization and post-hybridization washing buffers, and the pH was increased from 7.5 to 10. Moreover, EDTA, a strong cation and in particular Ca_2^+ chelator, was totally removed from all steps of this protocol. (4) Signal amplification. The TNT-1 (Tris-HCl, NaCl, and Tween 20) equilibration buffer, required for an optimal reaction of HRP with its substrate tyramide coupled with FITC, was adjusted to pH 9 and to a final concentration of 10mM Na_2CO_3. The duration of the tyramide signal amplification reaction was increased to 40min in order to compensate for the new, suboptimal buffer conditions. To stop the reaction, the pH and the Na_2CO_3 concentration of the TNT-1 buffer were adjusted to 10 and 10mM, respectively (TNT-2). (5) Filter preparation. The filters were rinsed for 10min in a 10mM Na_2CO_3 (final concentration) solution and counterstained with DAPI. Filters were then dried on a microscope slide at 35º C and soaked in commercial immersion oil (Olympus 04 Japan,OlympusOptical Co. Ltd., Tokyo, Japan). This oil was also used as an anti-fading agent, rather than the suggested AF3 reagent (citifluor) that dissolves the calcareous coccoliths. Slides were finally covered with a coverslip, sealed with transparent nail varnish, and kept at 4º C until observation, for a maximum of 2 weeks without loss of fluorescence.

Sample observation. Enumeration and taxonomic identification of the cells were performed at x 100 magnification by epifluorescence microscopy. We used an Olympus BX51 microscope (Olympus Optical Co. Ltd., Tokyo, Japan) equipped with a mercury light

source and a x 40 UV fluorescent objective. Excitation/emission filters were respectively 490/515nm for FITC staining and 360/420 nm for DAPI staining. For each microscope field, and immediately after epifluorescence counting, coccolith-bearing cells were detected and counted using visible light and cross-polarization, which is available on any basic Nomarski setup. Images were acquired with an RT-Slider Spot cooled charge coupled device digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) with constant exposure time and gain. The same filters were used for subsequent SEM characterization of the cell covers. The coverslip was carefully detached using a scalpel, immersion oil was washed out of the filters during two 10min baths in 5mL TNT-2 buffer at 37º C, and the filters were then rinsed for 10min in 6mM Na_2CO_3 at room temperature. Once dried, the filters were mounted on an aluminum support and coated with gold/palladium. Preparations were observed with a Jeol JSM 5200 LV SEM (JEOL, Tokyo, Japan).

3. Results

COD-FISH allows morphogenetic characterization of single coccolithophore cells. We first tested the applicability of the COD-FISH protocol in different life cycle stages of the coccolithophore species *E. huxleyi*, *Calcidiscus leptoporus*, and *Scyphosphaera apsteinii* (Fig. 18). The morphological integrity of the cells was maintainednin all studied species, not only at the end of the protocol but also after the retrieval of the filters for SEM (Fig. 18, D3 and E3). Even though some coccospheres collapsed, probably due to the initial filtration step, their architecture in general was well preserved, and no apparent sign of coccolith destruction and/or dissolution was observed. This result was achieved through fine tuning and optimization of pH, carbonate chemistry, and temperature along all steps of the COD-FISH protocol. Whenever possible, carbonate hypersaturation (Na_2CO_3 concentration $\geq$ 6mM), highly basic conditions (pH 10), and relatively low temperature were maintained in order to avoid carbonate dissolution. However, such conditions were not suitable for certain steps. For instance, the HRP enzymatic activity for signal amplification is repressed at pH $\geq$ 9. We thus used a pH of 9 but increased the final Na_2CO_3 concentration to 10mM for this particular step. A pH of 10 was restored immediately afterwards in the TNT-2 buffer that inactivates the enzyme. During the optimization process, we realized that pH values $\leq$ 9 combined with temperatures $\geq$ 37º C were leading to partial or total dissolution of the coccoliths, independent of Na_2CO_3 concentration. However, the pH of 7.5 applied during the fixation step, a necessary condition for efficient fixation, was not destructive. This may be due to the combination of low temperature (4º C) and relatively high

Na_2CO_3 concentration (6mM) used during this crucial step. In addition to coccosphere/coccolith preservation, the specificity of the PRYM02 probe for haptophyte algae was maintained despite the modifications to the original TSA-FISH protocol. The Chlorophyta and Heterokontophyta algae mixed with a culture of diploid *E. huxleyi*, and used as negative controls, were not targeted by the PRYM02 probe (Fig. 18 C).

COD-FISH as a quantitative tool. We performed culture tests to assess whether COD-FISH can be used as a quantitative method for the determination of cell densities of species or life cycle stages. These tests were based on mixtures of pure cultures of the two life cycle stages of *E. huxleyi*. We first estimated the cell densities of each pure culture by flow cytometry. Both cultures were then mixed, fixed, and filtered onto Anodisc membranes (see Methods). The filters where then processed for DAPI staining and CODFISH. At the end, cells counts were performed by epifluorescence and polarized microscopy. The three methods were thus quantitatively compared (Table 3). The strong consistency among the data of the different methods indicates that there were no detectable losses of either diploid or haploid cells during the COD-FISH process and that both life cycle stages were clearly distinguished when mixed assemblages were present.

Application of COD-FISH to natural populations. The final step was to test the robustness of our new detection and identification method in natural samples. For this purpose, we collected water from the Brittany coast (Roscoff, France) and during a mesocosm field experiment in Bergen, Norway. Samples were analyzed by COD-FISH, using the HRP-labeled probe PRYM02, and the filters were recovered for SEM analysis. In all samples, the fluorescently labeled cells were easily distinguishable from the clusters of naturally fluorescent particles typically present as background (Fig. 18, E2). Identification and enumeration of coccolithophores were performed under polarized visible light. In the samples from Roscoff, used for qualitative tests, many noncalcified haptophytes cells were detected, together with calcifying coccolithophores (data not shown). In the samples from Bergen, the COD-FISH counts in CPM (coccospheres of the largely predominant species *E. huxleyi*) were comparable to the flow-cytometric count data for *E. huxleyi* (Fig. 19). However, the COD-FISH fluorescent (FITC) quantification resulted in significantly higher numbers, as the PRYM02 probe targeted all other haptophyte algae present in the seawater sample. This revealed the presence of a high number of additional, noncalcifying haptophyte cells in the mesocosm bags. Within the context of the COD-FISH technique, development of a probe specific for *E. huxleyi* would allow determination of the proportion of these noncalcifying haptophytes that were in fact the haploid stages of this coccolithophore species.

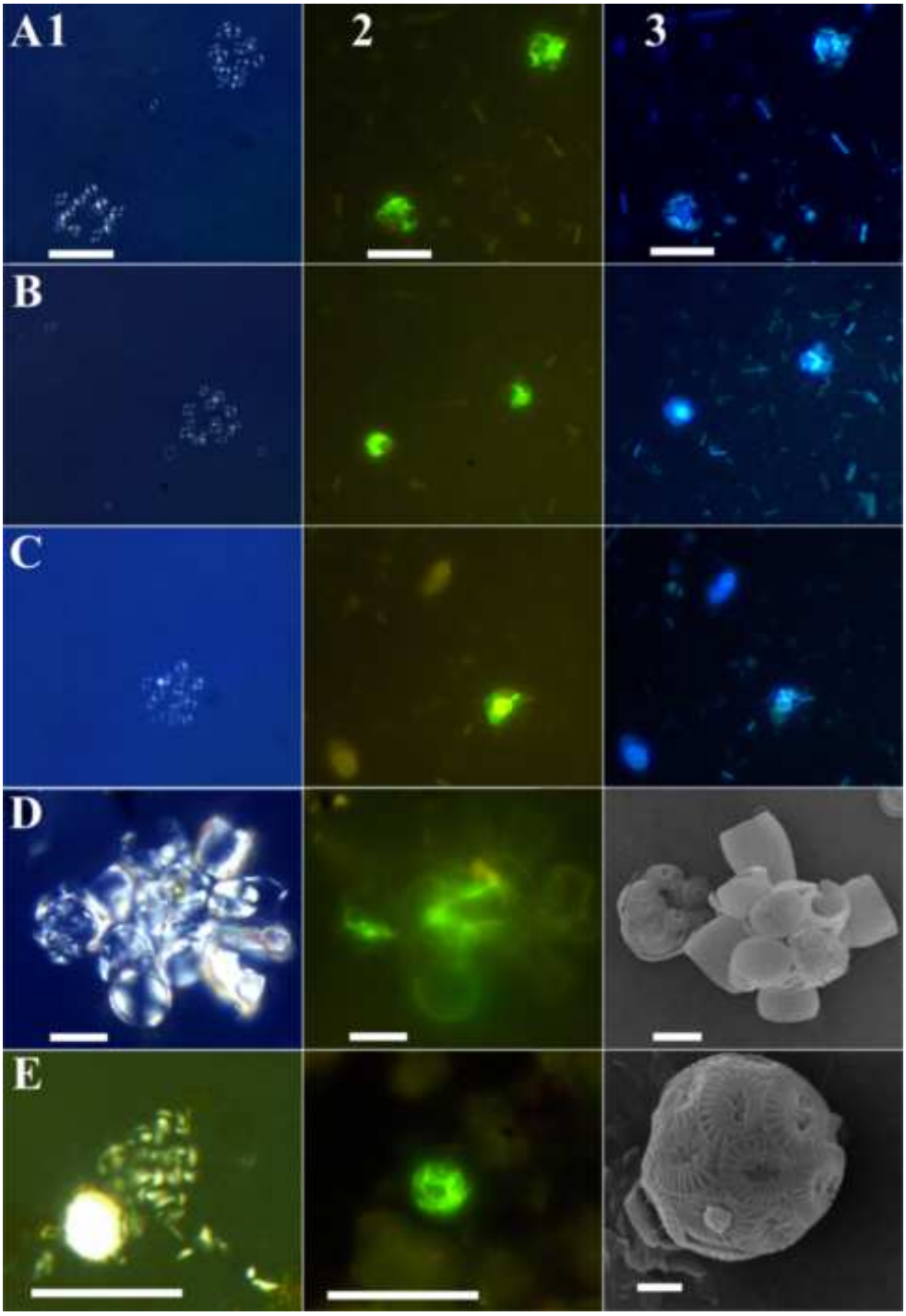


Figure 18. $CaCO_3$ optical detection with fluorescent in situ hybridization (COD-FISH) data. Parallel cross-polarized microscopy (CPM), tyramide signal amplification fluorescent in situ hybridization (TSA-FISH), and SEM analyses of several coccolithophores from unialgal and mixed cultures, as well as natural samples. Rows A–E, respectively, depict identical microscopy fields. (A) (1) CPM, (2) FITC, and (3) DAPI of a diploid culture of *Emiliania huxleyi*. (B) Like in A, but using a mixed, haplo-diploid culture of E. huxleyi. (C) Like in A, but using a mixed culture of diploid stages of *E. huxleyi* and other non-haptophyte microalgae, Micromonas sp., *Chlamydomonas concordia*, and *Pelagomonas calceolata*. (D) (1) CPM, (2) FITC, and (3) SEM of a mixed culture of the diploid stage of *Calcidiscus leptoporus* and the diploid, heterococcolith-bearing stage of Scyphosphaera apsteinii. (E) (1) CPM, (2) TSA-FISH, and (3) SEM of natural plankton communities collected offshore of, Roscoff, December 2004. COD-FISH revealed the presence of diploid specimens of *E. huxleyi*. Scale bars, 10 mm except for the SEM image E3 (1 mm).

Table 3. Comparison between counts (cells. mL^{-1}) performed by flow cytometry (using liquid fresh cultures), DAPI, and COD-FISH (after fixation and filtration of the same cultures).

	Flow Cytometry		**DAPI**		**COD-FISH**	
Life Cycle stages	Average[a]	Standard deviation	Average[b]	Standard deviation	Average[c]	Standard deviation
Diploid	1.76×10^6	7.64×10^4	2.05×10^6	1.46×10^5	1.74×10^6	3.19×10^5
Haploid	2.36×10^6	9.93×10^4	2.32×10^6	2.13×10^5	2.13×10^6	2.48×10^5

Pure culture of the diploid and haploid stages of *Emiliania huxleyi* strain AC472 were used in this experiment.
[a]Two replicates.
[b]Four replicates.
[c]Six replicates.

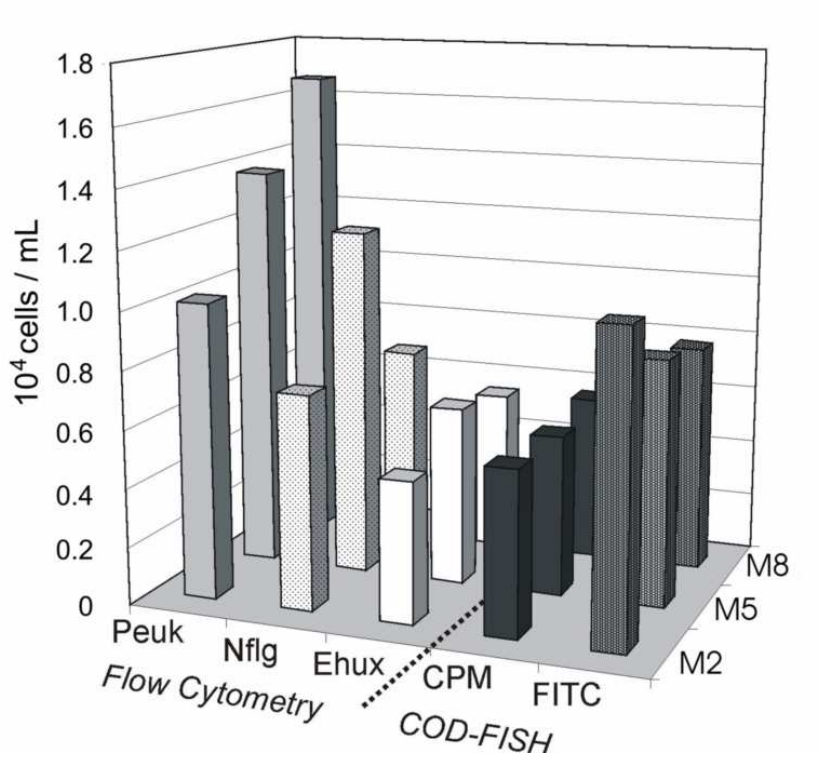


Figure 19. Flow cytometry versus $CaCO_3$ optical detection with fluorescent in situ hybridization (COD-FISH, using the Prymnesiophyceae- specific probe PRYM02) counts of planktonic populations in three mesocosm samples (bags, M2, M5, M8). Samples were collected in the exponential phase of the bloom. Peuk, pico-eukaryotes; Nflg, nannoflagellates; Ehux, *Emiliania huxleyi*; CPM, cross-polarization microscopy; FITC, epifluorescence microscopy with an excitation/emission at 490/515 nm.

4. Discussion

We developed a new protocol, COD-FISH, which allows quantitative morphogenetic assessment of coccolithophore cells collected from the ocean. The main challenge was to preserve their delicate, tiny, and composite calcareous coccoliths along the multiple steps necessary for the intracellular penetration, specific hybridization, and visualization of fluorescent rDNA probes. Every step of the classical TSA-FISH protocol was potentially destructive for the coccoliths and was thus finely tuned to avoid $CaCO_3$ dissolution. We optimized the pH, alkalinity, and temperature conditions while taking into account the enzymatic and biochemical constraints at each step. In addition, we were able to maintain the integrity of the composite skeleton of the coccolithophores (i.e. the coccosphere made of multiple coccoliths). This was achieved by coating the FISH filters with melted gelatin. The gelatin also served to prevent cell losses from the membranes after the PFA–ethanol fixation-permeabilization step. Importantly, the gelatin did not affect the efficiency of any subsequent steps of the protocol, even the SEM analysis of fluorescently labeled cells. COD-FISH was successfully tested on several species and life cycle stages of coccolithophores, representing a cross-section of the diversity within the group in terms of taxonomy, cell size, and perhaps most significantly skeleton morphology, including calcifying and noncalcifying forms. The penetration of HRP-labeled probes and their visualization was successful for all kinds of coccospheres: single or multiple layers of heterococcoliths, as well as holococcoliths.

The COD-FISH protocol has significant potential for the study of the biodiversity and ecology of coccolithophores. In the mesocosm experiment for instance, the use of COD-FISH with a haptophyte rDNA probe allowed rapid and simultaneous assessment of the numbers of calcifying haptophytes (mainly *E. huxleyi* in this case) and total haptophyte abundance. A simple subtraction of the number of *E. huxleyi* counted under CPM from the total number of fluorescently labeled cells gives the number of non-calcifying haptophytes, which accounted for between 1.2 (mesocosm 8) and 1.8 (mesocosms 2 and 5) times the amount of calcified diploid *E. huxleyi*. In this case, the fluorescent cells invisible under polarized light are either haploid, non-calcifying stages of *E. huxleyi* (Jacquet et al. 2002), and/or any other non-calcified haptophytes (Castberg et al. 2001). With the use of appropriate specific oligonucleotidic probes, COD-FISH can potentially be used to identify and quantify calcifying and non-calcifying stages of coccolithophores in natural samples at any taxonomic level. We predict that the use of COD-FISH with species-specific probes will go a long way toward resolving life cycle affiliations (and thus taxonomy) and ecology within the coccolithophores.

Finally, COD-FISH is not restricted to coccolithophores but can be applied to other microcalcifiers, such as the foraminifera or some dinoflagellates (e.g. *Thoracosphaera*). In fact, the development of such single-cell, morphogenetic protocols are crucial for our understanding of carbon cycling in the open oceans. Most $CaCO_3$ precipitation is driven by unicellular organisms characterized by extremely rapid generation times and rates of population turnover, and potentially high flexibility in terms of life cycle strategy. Until now, most studies of pelagic microcalcifiers have ignored their life as naked cells. Switches between life cycle stages may be, however, the most important biological feature driving carbonate chemistry and carbon fluxes in the pelagic environment. Future application of COD-FISH to widely distributed samples should provide the means to address these issues.

5. Aknowledgements

We would like to express our warm thanks to Daniel Vaulot who trustfully opened the door of his lab and dispensed advice. In addition, many thanks to: Nathalie Simon, Mário Cachão, Aude Houdan, and Joana Barcelos e Ramos who shared their phytoplankton expertise and helpful discussions; Telmo Nunes for technical assistance in SEM; and Ulf Riebesell, Aud Larsen, and the pelagic ecosystem CO2 enrichment study, PEECE, which provided the mesocosm samples and associated flow-cytometric counts. This work was supported by a US NSF grant DEB-0415351 (CdV, IP); an ATIP grant awarded to CdV by the ‘‘Centre National de la Recherche Scientifique,’’ France; and a Ph.D. fellowship SFRH/BD/16851/2004 awarded to MF by the ‘‘Fundação Para a Ciência e a Tecnologia,’’ Portugal. This work is part of the pluridisciplinary project BOOM (Biodiversity of Open Oceans Microalcifiers), funded by the ‘‘Agence National de la Recherche.’’

6. References

Amann R, Fuchs BM, Behrens S (2001) The Identification of Microorganisms by Fluorescence In Situ Hybridization. Current Opinion in Biotechnology 12:231-236

Amann RI, Ludwig W, Schleifer K-H (1995) Phylogenetic Identification and In Situ Detection of Individual Microbial Cells Without Cultivation. Microbiological Reviews 59:143-169

Biegala IC, Kennaway G, Alverca E, Lennon J-F, Vaulot D, Simon N (2002) Identification of Bacteria Associated With Dinoflagellates (Dinophyceae) Alexandrium ssp. Using Tyramide Signal Amplification- Fluorescent In Situ Hybridization and Confocal Microscopy. Journal of Phycology 38:404-411

Billard C (1994) Life cycles. In: Green JC, Leadbeater BSC (eds) The Haptophyta Algae, Vol 51. Clarendon Press, Oxford, p 167-186

Castberg T, Larsen A, Sandaa RA, Brussard CPD, Egge JK, Heldal M, Thyrhaug R, van Hannen EJ, Bratbak G (2001) Microbial population dynamics and diversity during a bloom of the marine coccolithophorid *Emiliania huxleyi* (Haptophyta). Marine Ecology Progress Series 221:39-46

de Vargas C, Probert I (2004) New keys to the Past: Current and future DNA studies in Coccolithophores. Micropaleontology 50:45-54

de Vargas C, Saez AG, Medlin LK, Thierstein HR (2004) Super-Species in the Calcareous Plankton. In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Delille B, Harlay J, Zondervan I, Jacquet S, Chou L, Wollast R, Bellerby RGJ, Frankignoulle M, Borges AV, Riebesell U, Gattuso JP (2005) Response of primary production and calcification to changes of pCO2 during experimental blooms of the coccolithophorid *Emiliania huxleyi*. Global Biogeochemical Cycles 19

Houdan A, Billard C, Marie D, Not F, Saez A, Young G, Probert I (2004) Holococcolithophores-heterococcolithophores (Haptophyta) life cycles: flow cytometry analysis of relative ploidy levels. Systematics and Biodiversity 1:453-465

Jacquet S, Heldal M, Iglesias-Rodriguez D, Larsen A, Wilson W, Bratbak G (2002) Flow cytometric analysis of an *Emiliana huxleyi* bloom terminated by viral infection. Aquatic microbial ecology 27:111-124

Keller MD, Selvin RC, Claus W, Guillard RRL (1987) Media for the culture of oceanic phytoplankton. Journal of Phycology 23:633-638

Malin G, Steinke M (2004) Dimethyl Sulfide Production: What is the Contribution of the Coccolithophores? In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Marie D, Brussaard CPD, Thyrhaug R, Bratbak G, Vaulot D (1999) Enumeration of marine viruses in culture and natural samples by flow cytometry. Appl. Environ. Microbiol. 65:45-52

Moon-Van der Staay SY, Van der Staay GWM, Guillou L, Claustre H, Medlin LK, Vaulot D (2000) Abundance and diversity of Prymnesiophyceae in the picophytoplankton community from the equatorial Pacific Ocean inferred from 18S rDNA sequences. Limnol. Oceanogr. 45:98-109

Not F, Simon N, Biegala IC, Vaulot D (2002) Application of fluorescent in situ hybridization coupled with tyramide signal amplification (FISH-TSA) to assess eukaryotic picoplankton composition. Aquatic Microbial Ecology 28:157-166

Rost B, Riebesell U, Burkhardt S, Sultemeyer D (2003) Carbon acquisition of bloom-forming marine phytoplankton. Limnology and Oceanography 48:55-67

Saez AG, Probert I, Geisen M, Quinn P, Young JR, Medlin LK (2003) Pseudo-cryptic speciation in coccolithophores. Proceedings of the National Academy of Sciences 100:7163-7168

Schönhuber W, Fuchs B, Juretschko S, Amann R (1997) Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. Appl. Environ. Microbiol. 63:3268-3273

Simon N, Campbell L, Erla Örnlfsdttir E, Groben R, Guillou L, Lange M, Medlin LK (2000) Oligonucleotide Probes for the Identification of Three Algal Groups by Dot Blot and Fluorescent Whole-Cell Hybridization. The Journal of Eukaryotic Microbiology 47:76-84

Stoeck T, Fowle WH, Epstein SS (2003) Methodology of protistan discovery: from rRNA detection to quality scanning electron microscope images. Appl. Environ. Microbiol. 69:6856-6863

Tyrrell T, Merico A (2004) *Emiliania huxleyi*: bloom observation and the conditions that induce them. In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Young J, Geisen M, Cros L, Kleijne A, Sprengel C, Probert I, Østergaard J (2003) A Guide to Extant Coccolithophore Taxonomy. Journal of Nannoplankton Research

Young JR, Didymus JM, Bown PR, Prins B, Mann S (1992) Crystal assembly and phylogenetic evolution in heterococcoliths. Nature 356:516-518

CHAPTER 3. *In situ* survey of the calcified and non-calcified life cycle phases of the coccolithophore *Emiliania huxleyi* (Prymnesiophyceae)

In situ survey of the calcified and non-calcified life cycle phases of the coccolithophore *Emiliania huxleyi* (Prymnesiophyceae)

Miguel Frada[1,2,*], Ian Probert[1], Peter Von Dassow[1], Willie H. Wilson[3], Daria Hinz[4], Colomban de Vargas[1]

[1]CNRS, UMR 7144 & UPMC Univ Paris 06, Equipe *EPPO* - Evolution du Plancton et Paléo-Océans, Station Biologique de Roscoff, 29682, France

[2]Centro de Geologia, Departamento de Geologia, Faculdade de Ciências, Universidade de Lisboa, Edifício C6, Campo Grande, 1749-016 Lisboa, Portugal

[3]Bigelow Laboratory for Ocean Sciences, 180 McKown Point, P.O. Box 475, West Boothbay Harbor, ME 04575-0475

[4]School of Ocean and Earth Science, National Oceanography Center, Southampton, University of Southampton, European Way, Southampton SO14 3ZH, UK

*corresponding author:

Miguel Frada
Station Biologique
Equipe EPPO - Evolution du Plancton et Paléo-Océans (UMR 7144)
Place Georges Teissier, Roscoff (France)
Phone : 33 (0) 2-98-29-25-28
email : frada@sb-roscoff.fr

In preparation : **Aquatic Microbial Ecology**

Abstract

Emiliania huxleyi is the most intensively studied coccolithophore. However, the quasi-totality of information on this species concerns exclusively one morphological type of cells within its life cycle: the calcified diploid stage. Very little is known about the biology and ecology of non-calcified stages, which may be non-motile and diploid or motile and haploid. Using a fluorescent in situ hybridisation approach coupled with cross-polarized microscopy, we revealed the presence of non-calcified *E. huxleyi* cells in various coastal and oceanic environments. These cells formed background populations generally at much lower concentrations than the calcified morphotypes, with dynamics paralleling those of the calcified diploid cells. In a mesocosm experiment where a bloom of diploid calcified *E. huxleyi* was artificially induced, non-calcified cells were present from the outset and remained at low concentrations until the virally-induced termination of the bloom, at which point a small peak of non-calcified cells was recorded. This population comprised both non-calcified diploid cells and haploid swimming cells. We conclude that although diploid calcified cells largely predominated natural populations of *E. huxleyi*, non-calcified cells were always present. The ecological role of non-calcified cells is still uncertain, but they may constitute a continuous seed-bank of cells that actively participates in the ecological dynamics of *E. huxleyi* by re-inseminating calcifying populations, being thus necessary for the survival of the species.

Keywords: Coccolithophores, *Emiliania huxleyi*, life cycle; mesocosm; open ocean

1. Introduction

Emiliania huxleyi is the most abundant and ubiquitous extant coccolithophore, constituting an important component of phytoplankton assemblages (McIntyre & Bé 1967, Campbell et al. 1994). This species is capable of forming blooms that cover vast oceanic and neritic areas (Naninga and Tyrrel 1996). During the termination of *E. huxleyi* blooms the calcareous scales (coccoliths) shed from virally-infected and dying cells scatter sunlight resulting in wide patches of milky turquoise waters that are readily observed in satellite images (Holligan et al. 1993, Brown & Yoder 1994, Nanninga & Tyrrell 1996, Wilson et al. 2002, Tyrrell & Merico 2004). This massive development of *E. huxleyi* populations has significant environmental and climatic impacts (Westbroek et al. 1993, Malin & Steinke 2004, Rost & Riebesell 2004). As a result, the morpho-genetic diversity, ecophysiology and biogeochemistry of *E. huxleyi* have been intensively studied in both laboratory-based and field experiments (see (Paasche 2001)and references therein) and a whole genome sequencing project is nearing completion (http://genome.jgi-psf.org/).

One of the basic biological features of *E. huxleyi*, shared with all prymnesiophytes (Billard 1994), is the possession of a haplo-diplontic and heteromorphic life cycle. Studies performed with culture strains (Klaveness 1972, Green et al. 1996) have described *E. huxleyi* as exhibiting three main life phases, all capable of independent mitotic multiplication: a diploid, coccolith-bearing, non-motile and bloom-forming phase (calcifying or "C-cell'); a diploid, non-calcified, non-motile phase ('naked -cell'); and a haploid, non-calcified, biflagellated phase, bearing organic scales bound to the plasmalema (scaly or 'S-cell'), and which likely serves at some point as gametes.

Beyond this basic information, the vast majority of studies on this species concern exclusively the diploid coccolith-bearing phase, due largely to the fact that non-calcified cells are not easily identifiable using conventional light and electron microscope techniques. However, a recent *in vitro* study reported that the calcified diploid phase and the non-calcifying motile haploid phase have comparable growth rates, both being able to form dense populations in pure culture, but they display different photophysiologies, since photosynthesis of diploid cells remained efficient over a range of light intensities, whereas the haploid cells were photoinhibited at high light levels (Houdan et al. 2005). These differences led the authors to suggest the existence of spatial and/or temporal niche differentiation in nature, as has been proposed for other coccolithophore species (Cros et al. 2000, Houdan et al. 2006). in another comparative study (Frada et al. 2008) it was demonstrated that the haploid stage is not

infected by the lytic viruses which are known to decimate *E. huxleyi* diploid calcifying blooms (Bratbak et al. 1993, Wilson et al. 2002, Martinez et al. 2007). Non-calcifying cells may thus act as resistant stages allowing survival of the species in the face of massive viral infections. *In vivo*, a single study has reported the presence of non-calcified *E. huxleyi* cells in the plankton by means of immunochemical labelling, resulting in total *E. huxleyi* counts (Campbell et al. 1994). However, the proportions and dynamics of calcified versus non-calcified cells have never been studied on large temporal and/or spatial scales, and thus the prevalence and role of non-calcified phases in wild populations remains completely unknown.

Our aims in this study were to directly detect and enumerate calcified and non-calcified cell of *E. huxleyi* in field samples covering different ecological settings and population dynamics. Moreover, we attempted to unveil the ploidy level of the non-calcified cells. This information is highly relevant in the contexts of further understanding the population dynamics, diversity and evolution, as well as the biogeochemical impact of this important phytoplankton, taking into account all life cycle stages of this species.

2. Material and Methods

2.1. Samples and sampling sites

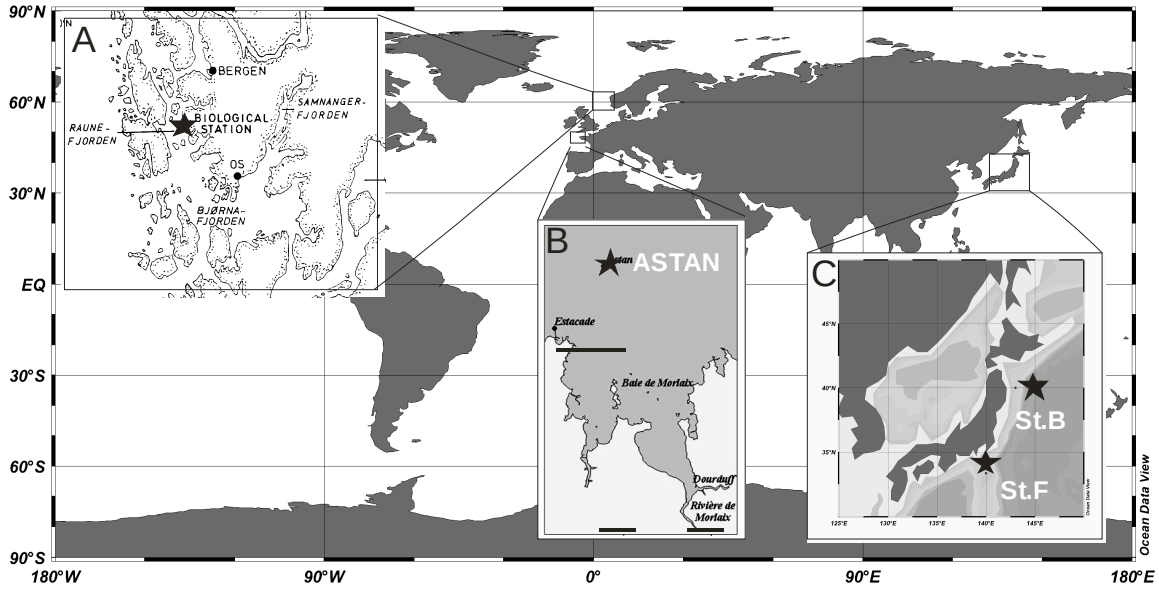


Figure 20. Location of the sampling sites. A. Mesocosm (Norway); B. ASTAN-SOMLIT station, off-shore Roscoff (France); C. West Pacific Station.

Mesocosm

The mesocosm experiments designed to monitor the progression of an induced *E. huxleyi* bloom were carried out in Raunefjord in western Norway at the Marine Biological Field Station at Espeland (Fig. 20 A), 20 km south of Bergen during June 2008. Six transparent polyethylene enclosures (11 m^3; 90% penetration of photosynthetically active radiation) purchased from ANI-TEX (Notodden, Norway) were mounted on floating frames moored along the south side of a raft (for details, see (Egge & Heimdal 1994)). Enclosures were filled on June 2nd with unfiltered seawater from a point adjacent to the raft at a depth of 2 m with a water-pump. The seawater in the enclosures was kept homogeneous by means of airlifts and enriched daily (between 12 am and 4 pm) with nitrate (added as $NaNO_3$) and phosphate (added as K_2HPO_4) in order to induce an *E. huxleyi* bloom. Three mesocoms were enriched at a non-limiting N to P ratio (1.5 μM $N\text{-}NO_3$:0.1 μM $P\text{-}PO_4$) and three were limited in phosphate (1.5 μM $N\text{-}NO_3$:0.02 μM $P\text{-}PO_4$) (for details see (Castberg et al. 2001, Jacquet et al. 2002)).

Before the mesocosm experiment, water samples were collected from the fjord adjacent to the mesocosm raft. These samples were collected with a Niskin bottle from 3 depths (0, 5 and 10 m). During the mesocosm experiment, samples were collected from all 6 bags or enclosures (only results from enclosure 2 and 3 are shown here) with a 10L bottle every day for 17 days at 6 am. Between days 0 and day 5, 50 and 200 ml of water were processed for COD-FISH and for cellular DNA content analysis (see below); between days 6 and 12, 20 and 100 ml of water were processed; and during the last days 5 and 50 ml of water were processed. Samples for transmission electronic microscopy and flow cytometry were also collected daily (see below).

Raunefjord

Samples from the Raunefjord (off-shore from the Marine Biological Station, University of Bergen) south of Bergen (Fig. 20 A) were collected on the 15th May and 1st June 2007. Water samples were collected with a Niskin bottle from the surface. 150 to 250 ml of seawater were used for further treatments (see COD-FISH below).

English Channel

Samples from the English Channel (Fig. 20 B) were collected in the SOMLIT-ASTAN station (off Roscoff, France: 48°46′ N, 03°57′ W) at 1m depth with regular bottle every other week throughout the year 2007. 150 to 500 ml of seawater were used for further

treatments (see COD-FISH below). Data for temperature and Chl *a* concentration were provided by the 'Service d'Observation en Milieu Littoral, INSU-CNRS, Station Biologique de Roscoff' (http://www.domino.u-bordeaux.fr/somlit_national/).

West Pacific (off-shore Japan)

Samples from the west Pacific (Fig. 20 C) were collected during the KT06-11 cruise on board the R/V Tansei Maru (JAMSTEC, Japan) in June 2006. Water column profiles from two oceanic stations (B: 40ºN, 145ºE; F: 34º26'N, 139º03'E) were collected at different depths with a rosette sampler coupled to a CTD/fluorometer. 300 to 600 ml of seawater from each depth were used for further treatments (see COD-FISH below).

2.2. Light and epifluorescence microscopic observation of fresh samples

Unfixed samples from the mesocosm were monitored every day by light and epifluorescence microscopy (Leica DMR Type 020-525.024). Image acquisition was performed with a standard digital camera (SONY cyber-shot 5.1 mega pixels) directly through the microscope eyepiece.

2.3. COD-FISH

To detect and enumerate *Emiliania huxleyi* cells in natural samples, we used the $CaCO_3$ Optical Detection with Fluorescent *In Situ* Hybridization method (COD-FISH) ((Frada et al. 2006). This quantitative method couples fluorescent *in situ* hybridization with cross-polarizing microscopy, allowing the simultaneous assessement of a given taxonomic coccolithophore group (or species) through the utilization of specific oligonucleotidic probes, and determination of the morphological status of single cells, including coccolithophore life cycle phases that are generally characterised by a differential morphology (Billard 1994). In this work, we used this method to assess and monitor calcified and non-calcified *Emiliania huxleyi* cells in natural samples.

2.3.1. Probe design and specificity testing

A 28S rDNA-specific oligonucleotide probe, EG28-03, (5'-TAAAGCCCCGCTCCCGGGTT-3') was developed to specifically target both *E. huxleyi* and the closely related sister species *Gephyrocapsa oceanica*. The probe was designed using the

ARB software ((Ludwig et al. 2004); http://www.arb-home.de) based on a database of 28S rDNA sequences of all haptophyte strains from the Roscoff Culture Collection (http://www.sb-roscoff.fr/Phyto/RCC). The specificity of the probe was tested *in silico* against the LSU reference database available on the SILVA website (http://www.arb-silva.de/). The oligonucleotide with a 5′-aminolink (C6) (Eurogentec, France) was labelled with horseradish peroxidase (HRP) as described in (Urdea et al. 1988, Amann et al. 1995).

Prior to the utilization of the probe in natural samples, the specificity of the EG28-03 probe was tested against several algae strains from the Roscoff Culture Collection (http://www.sb-roscoff.fr/Phyto/RCC/), including haptophytes (*E. huxleyi* RCC1216, including haploid non-calcified and diploid calcified, *E. huxleyi* RCC1249 including haploid non-calcified and diploid calcified, *Gephyrocapsa oceanica* RCC1315 including haploid non-calcified and diploid calcified, *Isochrysis galbana* RCC1347, *I. galbana* RCC1349, *Pleurochrysis carterae* RCC1402, *Algirosphaera robusta* RCC1128, *Syracosphaera pulchra* RCC1131, *Prymnesium* sp. RCC1348; *Chrysochromulina camella* RCC1186, *Phaeocystis globosa* RCC184, *Pavlova* sp. RCC1543), a cryptophyte (*Rhodomonas salina* RCC20), a diatom (*Skeletonema costatum* RCC70), a dinoflagellate (*Amphidinium carterae* RCC88) and a chlorophyte (*Dunaliella tertiolecta* RCC6). The haptophyte general probe Prym-02 (Simon et al. 2000) was used as a positive control for the haptophytes (following the protocol described in Frada et al., 2006).

2.3.2. *Application of the EG28-03 probe to field samples*

Sample collection, storage, hybridization, visualization and enumeration (in triplicate) of the cells followed the COD-FISH protocol described by Frada et al. (Frada et al. 2006) with minor modifications to account for the new probe: (1) all samples were fixed with 1% paraformaldehyde (PFA) as described in the original protocol, except samples taken in Japan, for which PFA was reduced to 0.1% in order to avoid the formation of precipitates; (2) hybridization was performed at 40ºC for 12h in 45% formamide hybridization buffer; (3) the washing step was performed at 42ºC with NaCl in the buffer adjusted to 51 mM; and (4) TNT buffer washes were performed at 42ºC.

We further compared our COD-FISH counts with the flow cytometric counts of the same samples made by Wilson et al. (in prep.) in order to verify the quantitative nature of our method in natural samples.

2.4. Determination of cellular DNA content by quantitative epifluorescent microscopy

A protocol was designed to measure the fluorescence intensity of nuclei stained with 4',6-diamidino-2-phenylindole (DAPI) (iDAPI, intensity of DAPI) of calcified and non-calcified *E. huxleyi* cells collected during the mesocosm experiment. These measurements of allow quantification of the realtive genome size and therefore can be used to estimate the relative ploidy level of individual cells. Measurements of the fluorescence intensity of whole cells were also performed in order to complement the nuclear staining analyses

The *E. huxleyi* strains RCC1216 and RCC1217 (calcified diploid and non-calcified haploid) were used to calibrate the method. After calibration, samples from the fjord prior to the beginning of the experiment (Fjord) and day 16 were analysed.

In practical terms, after processing the samples for COD-FISH, the anodisc filters (Whatman, Maidstone Kent, UK) were divided into two pieces. One half was washed for 10 minutes in 20 mM Na_2CO_3 500mM EDTA pH 7.5 to remove the coccoliths of calcified cells. Filters with haploid cells were also washed in this solution and used to control the effect of this treatment on preservation of cells and staining of nuclei. All filters were then incubated in a 20 mM Na_2CO_3 200ng ml^{-1} DAPI solution at 42ºC in the dark for 10 min. The filters were then dried at 50ºC in the dark and mounted in immersion oil (Olympus 04, Japan) between a glass slide and a coverslip. Observations were performed with a Zeiss Observer.Z1 microscope (Zeiss, Le Pecq France) using the DAPI (ex.359 nm; em.461 nm) and the FITC (ex.470 nm; em.509 nm) channels. Visualization of coccoliths was performed with Nomarski interference optics. Image acquisition was performed with no gamma corrections, constant gain and background correcion turned off, at 100% pixel intensity and fixed exposure time with an AxioCam MRm camera, coupled to AxioVision software (Zeiss, Le Pecq France).

Epifluorescence images were analyzed using ImageJ v. 1.40g (National Institute of Health, USA). In-focus nuclei and whole cells were delineated (Fig. 21), and the integrated density in the DAPI channel was measured and corrected using measurements of background fluorescence intensity from cell-free areas of the same image.

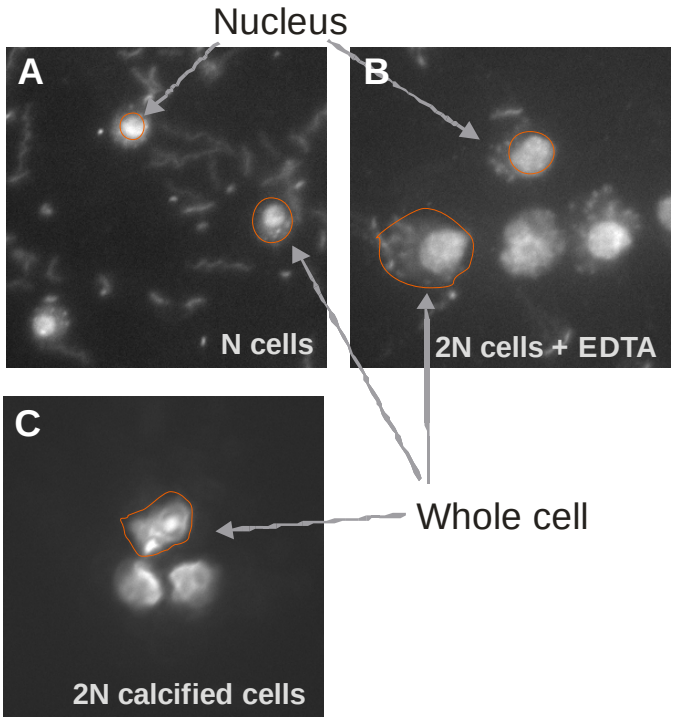


Figure 21. Analyses of the DAPI intensity (iDAPI) of whole cells and nuclei of *Emiliania huxleyi* cells. (A) haploid cells; (B) diploid cells treated with EDTA to remove the coccoliths; (C) diploid calcified cells. The red circle represent the limit of the area of the cells or nuclei analysed.

Statistical comparisons were performed two by two using the non-parametric Man-Whitney test, using the SYSTAT 10 software (SPSS Inc.). It should be noted that the culture samples were solely used to calibrate the method and no direct comparisons with the mesocom samples were performed mainly because of two issues: 1) the physiological state of cultured strains and wild cells likely differed and this could affect the brighthness of the nuclei; 2) DAPI staining is variable depending on several parameters, including small differences in the incubation time and background composition of the sample; 3) there is a significant variation in genome size between *E.huxleyi* strains (Probert et al., unpublished data). Therefore comparisions were made within culture samples and within mesocosm samples.

2.5 Transmission electronic microscopy (TEM)

Cells were harvested from 1 litre of mesocosm water by gentle filtration through a 47mm diameter 0.8µm pore size polycarbonate filter (Osmonics Inc, Minnetonka USA). The cells were then carefully resuspended by pippetting in the pre-fixative buffer: 2.5%

glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2) with 0.25 M sucrose. The remaining steps were performed as described in Frada et al. (Frada et al. 2008). Note that samples were stored for one month at room temperature in the dark at the 70% ethanol dehydration step. Observations were carried out on a Jeol 1400 microscope (Jeol Ltd, Tokyo Japan).

2.6 Flow cytometry analysis of viruses

Viral communities in all the mesocosm enclosures was performed daily by flow cytometry as described in Martínez et al. (Martinez et al. 2007).

3. Results

3.1. Specificity of the *Emiliania huxleyi* 28S rDNA probe EG28-03

The new probe hybridized positively, under our methodological conditions, with both *E. huxleyi* and the sister species *G. oceanica* (positively labelled cells were green in colour due to the fluorescein fluorochrome) (Fig. 22 A, B and C), and did not hybridize with any of the other haptophytes or other microalgae tested, these appearing dark or weakly visible due to autofluorescence (Fig. 22). This test confirmed the probe specificity and its applicability in natural samples containing a wide assemblage of organisms. Among the *E. huxleyi* and *G. oceanica* strains, only the diploid calcified cells were visible under cross-polarized light, due to the birefringence of the coccoliths.

Conversely, the Prym-02 probe labelled all haptophytes (and no other microalgae), demonstrating the good physiological state of the tested cultures. Some calcified strains (coccolithophores) were visible in cross-polarized light (Fig. 22 F, G, H).

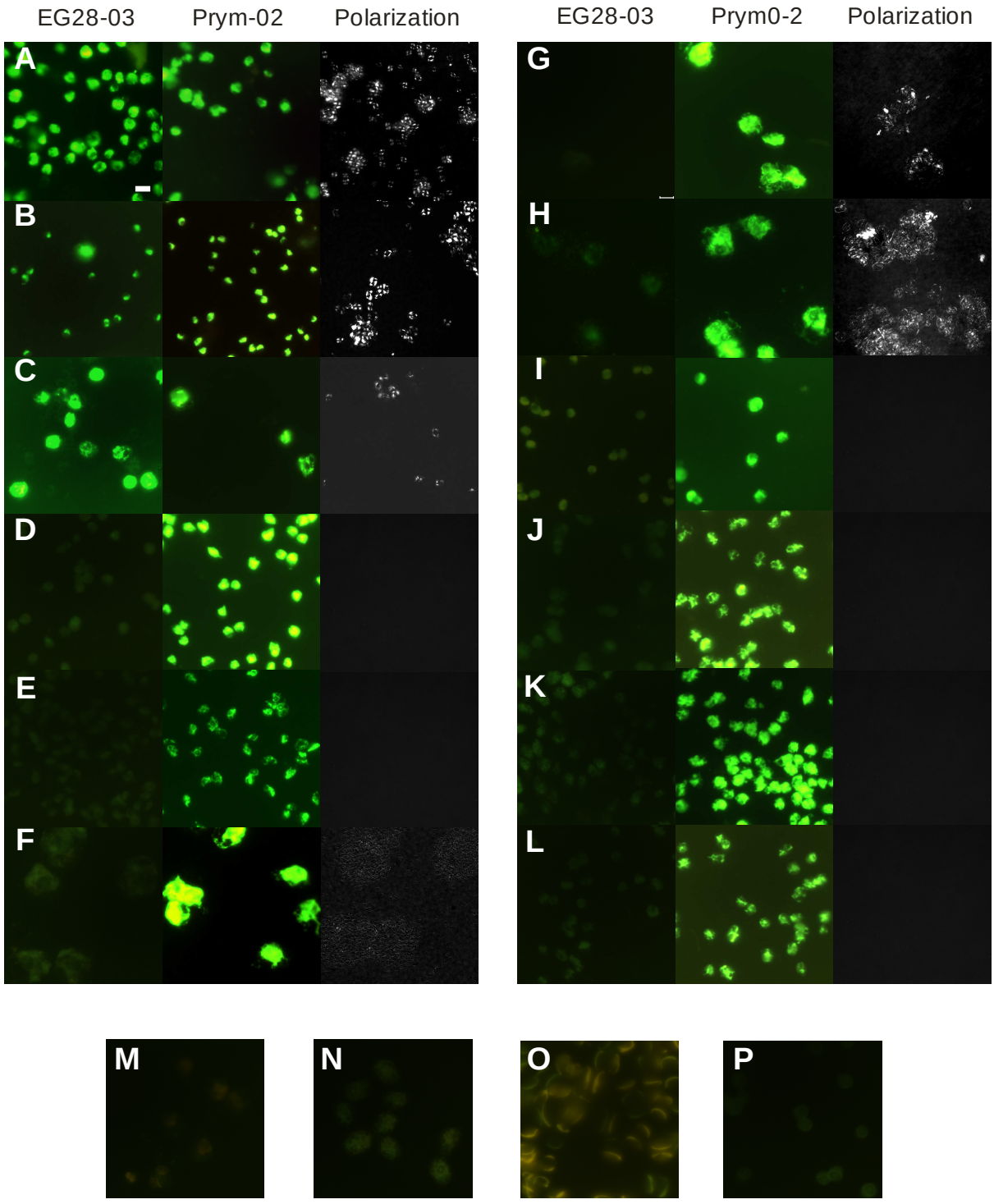


Figure 22. Specificity test of the probe EG28-03. All the tested strains are visualized by epifluorescence hybridized both with the EG28-03 and Pym-02 probes and by cross-polarization. **Haptophytes**: A. *Emiliania huxleyi* RCC1216 (mixture of diploid and haploid cells); B. *E huxleyi* RCC1249 (mixture of diploid and haploid cells); C. *Gephyrocapsa oceanica* RCC1315 (mixture of diploid and haploid cells); D. *Isochrysis galbana* RCC1347; E. *I. galbana* RCC1349; F. *Pleurochrysis carterae* RCC1402; G. *Algirosphaera robusta* RCC1128; H. *Syracosphaera pulchra* RCC1131; I. *Prymnesium* sp. RCC1348; J. *Chrysochromulina* sp. RCC1186; K. *Phaeocystis* sp. CCMP2496; L. *Pavlova* sp. RCC1543. **Non-haptophytes** (only tested with EG28-03): M. *Rhodomonas salina* RCC20; N. *Skeletonema costatum* RCC70; O. *Amphidinium carterae* RCC88; P. *Dunaliella tertiolecta* RCC6.

3.2. Population dynamics of *E. huxleyi* during the mesocosm experiment

3.2.1. Light and epifluorescence microscopy observations of fresh samples

In mesocosm samples, well-calcified *E. huxleyi* cells were observed at the beginning of the experiment (Fig.23 A) together with various flagellates, including dinoflagellates, cryptophytes and other flagellated haptophytes. No *Gephyrocapsa* spp. cells were detected and a single other coccolithophore, *Acanthoica* sp. (Rhabdosphaeracea, Fig. 23 B), was present at low concentrations throughout the experiment.

At days 8-9, *E. huxleyi* was largely majoritary, while other microalgae were rarely observed. Between days 13 and 16, in parallel with the end of exponential growth and beginning of the stationary phase of the calcified *E. huxleyi* population, some groups of 2-3 non-calcified flagellated cells with chloroplasts were observed adjacent to agglomerates of coccoliths (Fig. 23 C and D). On one occasion these cells were observed swimming away from the coccolith debris. During this period we also observed some *E. huxleyi* cells with the coccosphere semi-opened and losing their coccoliths. These cells increased abruptly in number on days 15 and 16. During this last period of the experiment we also observed the emergence of large *Chrysochromulina*-like haptophytes displaying long haptonema and well developed chloroplasts, as well as other small flagellates with no sign of chloroplast autofluorescence.

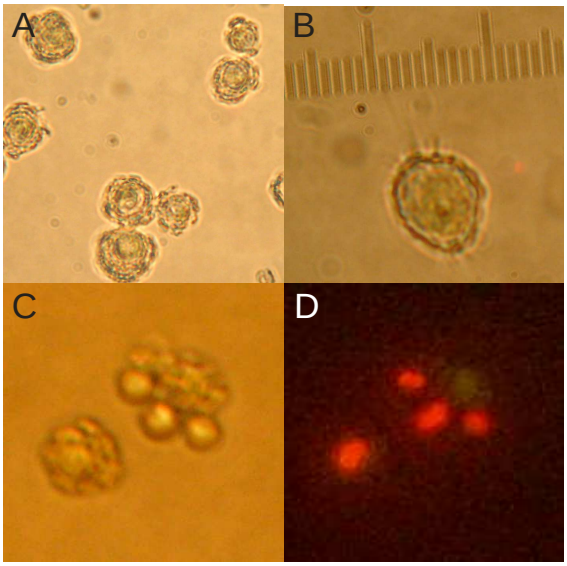


Figure 23. Light and epifluorescence microscopy images from fresh samples collected during the mesocosm bloom (scale absent). (A) *E. huxleyi* diploid cells. (B) *Acanthoica* sp. Cell. (C) Group of 3 non-calcified flagellated cells attached to an agglomerate of coccoliths and an isolated *E. huxleyi* calcified cells (day 15 bag 3). (D) UV light epifluorescence microcopy image showing the chloroplast autofluorescence of the non-calcified cells observed in C.

3.2.2. COD-FISH analyses

In the water column surrounding the mesocosm raft, calcified *E. huxleyi* cells (Fig. 24 A) were recorded with a relatively even vertical distribution at concentrations of approximately 3.0 x 10^2 cells ml^{-1} prior to the begining of the mesocosm experiment. Non-calcified *E. huxleyi* cells were also present (Fig. 25 A), but at lower concentrations (1.7 x 10^1 cells ml^{-1}), corresponding to 5 to 10% of total *E. huxleyi* population.

During the mesocosm experiment (Fig. 24 B), in both the P limited enclosure (bag 2) and the P and N replete enclosure (bag 3) the calcified *E. huxleyi* cells (Fig. 25 B) remained at low concentrations during a lag period of 5 to 7 days in both bags and then started growing exponentially at day 7, being visibly well calcified and labelled with the EG28-03 probe (Fig. 25 B and C) . The growth rates calculated during this phase were ~1.8 day^{-1} for bag 2 (days 7-12) and ~1.7 day^{-1} for bag 3 (days 7-13). In bag 3 the exponential phase was longer and the population attained approximately double the maximal concentration recorded in bag 2. Conversely, there was practically no stationary phase during the time-course of the experiment in bag 3, whereas in bag 2 the population was in stationary phase from day 12 until day 16. At the middle/end of the exponential phase of *E. huxleyi* population development, viral particles started proliferating in both bags, increasing exponentially to maxima of ~13.4 x 10^6 viral particles ml^{-1} at day 16 in bag 2 and ~28.3 x 10^6 viral particles ml^{-1} on day 15 in bag 3. In bag 2, the *E. huxleyi* population collapsed abruptly to approximatively 2.8 x 10^3 cells ml^{-1}, right after the viral peak. In bag 3, the cells started decreasing the day before the viral peak and then collapsed progressively to approximatively 2.0 x 10^3 cells ml^{-1} in 4 days.

The non-calcified *E. huxleyi* cells (Fig. 24 B, zoom) remained at very low concentrations in both bags until day 9 (approximately 1 to 2% of the whole *E. huxleyi* population). A progressive increase was then recorded, from day 10 until day 15, with these non-calcified populations rapidly peaking in both bags at day 16 (bag 2: 5.9 x 10^3 cells ml^{-1}; bag 3: 1.0 x 10^4 cells ml^{-1}). These rapid increases coincided with the last day of stationary phase of the calcified cell population in bag 2 and coincided with the decline of calcified cells in bag 3. At this point, non-calcified *E. huxleyi* cells accounted for ~ 9% (bag 2) and ~ 40% (bag 3) of the whole *E. huxleyi* populations. On day 17, like the calcified cells, the concentration of non-calcified cells decreased suddenly, but still accounted for ~23% and 33% of the whole *E. huxleyi* populations in bags 2 and 3, respectively. During these last days of the bloom, the detected non-calcified cells were always brighty labelled with the EG28-03 probe (Fig. 25 D to F).

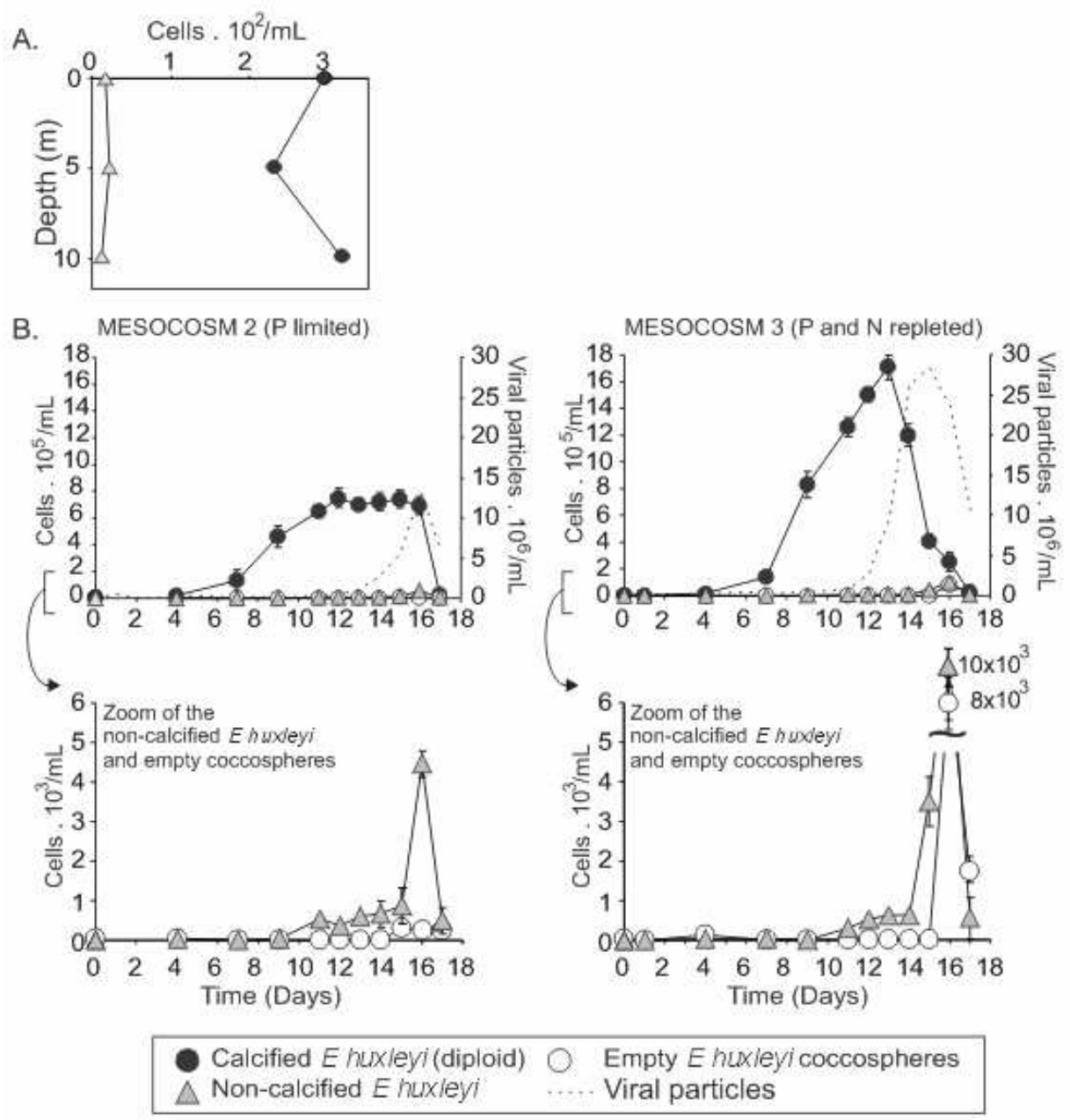


Figure 24. Dynamics of calcified and non-calcified *Emiliania huxleyi* populations, as well as viral particles during the mesocosm experiment.

E. huxleyi and viral particles were monitored by COD-FISH and flow cytometry respectively. (A) Concentration of calcified and non-calcified *E. huxleyi* cells in natural waters surrounding the mesocosm experiment on day 0. (B) Concentrations of calcified and non-calcified of *E. huxleyi* cells and viral particles in P-limited and nutrient-rich conditions. A zoom on population dynamics of non-calcifying cells and empty *E. huxleyi* coccospheres is given at the bottom.

During the COD-FISH survey of mesocosm populations, we also monitored the presence of empty *E. huxleyi* coccospheres (Fig. 24 B, zoom), lacking fluorescence of both the COD-FISH probe and DAPI stained nucleus (Fig. 24 B). These 'ghost' cells were absent in the fjord water surrounding the mesocosms, but were observed in the mesocosm bags at the

beginning of the experiment (from day 0 until day 4). They were then not observed until day 15. In bag 2 the highest concentration of empty coccospheres was observed on day 15 (3.9 x 10^2 empty coccospheres ml^{-1}) and in bag 3 at day 16 (8.5 x 10^3 empty coccospheres ml^{-1}). Then they declined at day 17 together with the cells.

Finally, very similar results were obtained (Supplementary information 1) throughout the mesocosm experiment from COD-FISH counts of the calcified *E. huxleyi* population and flow cytometric counts presented by Wilson et al. (in prep.).

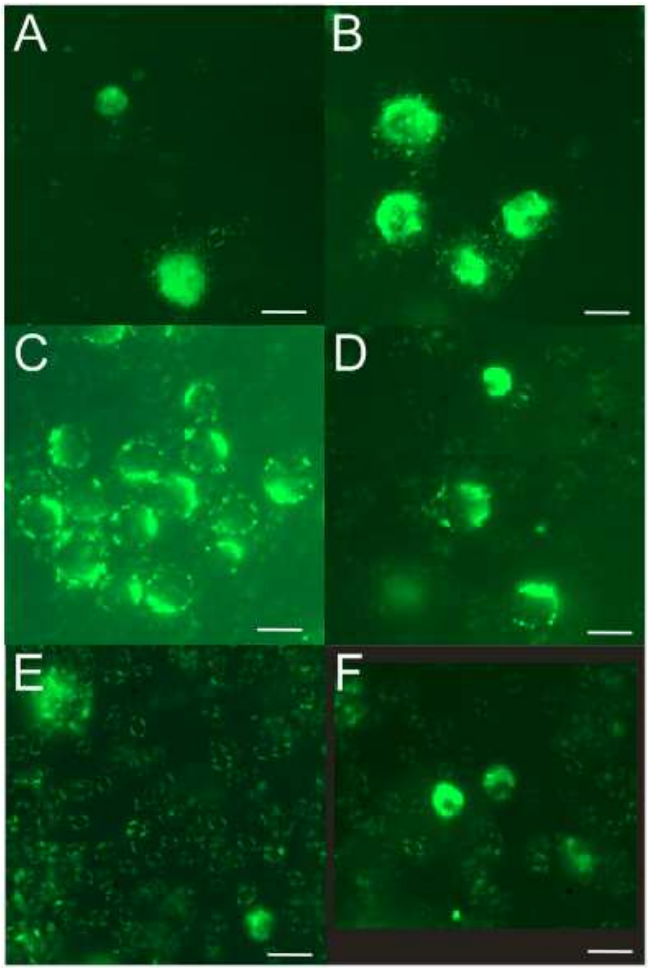


Figure 24. Merged images of epifluorescence and cross-polarization of *E. huxleyi* detected during the mesocosm (The epifluorescence detection was made with the EG28_03 probe).

(A) *E. huxleyi* non-calcified cell (top) and diploid cell surrounded by coccoliths (calcified) (bottom) – Day 0, bag 3; (B) *E. huxleyi* calcified cells – Day 0, bag 3; (C) *E. huxleyi* calcified cells – Day 9, bag 3; (D) *E. huxleyi* non-calcified (top) and two calcified cells (bottom) – Day 16, bag 3; (E) *E. huxleyi* non-calcified cell (bottom) and calcified cell (top) – Day 16, bag 2; (F) *E. huxleyi* non-calcified cells – Day 17, bag 3

3.2.3. Quantitative analysis of DAPI stained nuclei

Analysis of samples from cultured strains

Measurements of diploid calcified and decalcified (EDTA treated) whole cells gave comparatively similar results. The iDAPI of both means were statistically identical (~17 x 10^4 iDAPI), but the range of calcifed cells was wider as a result of the fluorescence scatter by the coccoliths covering the cell. Haploid cells, treated or untreated with EDTA, displayed

statistically similar means (~7.5 x 10^4 iDAPI) and also similar ranges, confirming that the EDTA treatment did not affect DAPI staining and intensity. Moreover, the mean iDAPI of diploid cells was twice as high relative to the iDAPI of haploid cells, and the difference between both types of cells was statistically significant (Fig. 26 A).

Concerning the nuclei, the discrimination of diploids and haploids were less marked. Diploid nuclei (only nuclei from EDTA decalcified cells) were on average twice as bright (~11 x 10^4 iDAPI) as haploid nuclei (untreated and EDTA treated) (~5 x 10^4 iDAPI) and the difference between both types of nuclei was statistically significant. However, the range of diploid and haploid cells overlapped. It should be noted also that the measurements of haploid nuclei treated with EDTA or untreated were statistically identical, revealing once again the inexistence of a negative effect of the EDTA treatment on DAPI staining and intensity (Fig. 26 B).

Analysis of the mesocosm samples

The same analysis was then performed on natural samples from day 16 and also from a sample collected in the fjord before the mesocosm experiment.

In the untreated samples of day 16, two populations of whole cells were detected: the diploid calcified cells (~16 x 10^4 iDAPI) and a population with lower average iDAPI brightness of non-calcified cells (~13 x 10^4 iDAPI) (Fig. 26 C, left). The mean of this last population of non-calcified cells was statistically similar to the mean of the cells measured in the EDTA-treated sample (Fig. 26 C, right), which comprises all types of *E. huxleyi* cells present in the sample. However, the range of iDAPI intensity of these group of cells was wider, encompassing the range of the non-calcified population (from the untreated samples) and a group of cells with brighter iDAPI fluorescence.

Concerning the nuclei (Fig. 26 D), the iDAPI mean of the non-calcified cells (untreated) was statistically similar to the nuclei of the EDTA-treated samples (~6 x 10^4 iDAPI). It should be noted however, that the range of nuclei intensities was wider in EDTA-treated samples comprising, like in whole cells, the range of the nuclei of the non-calcified population and a population with higher iDAPI fluorescence that was present in the water.

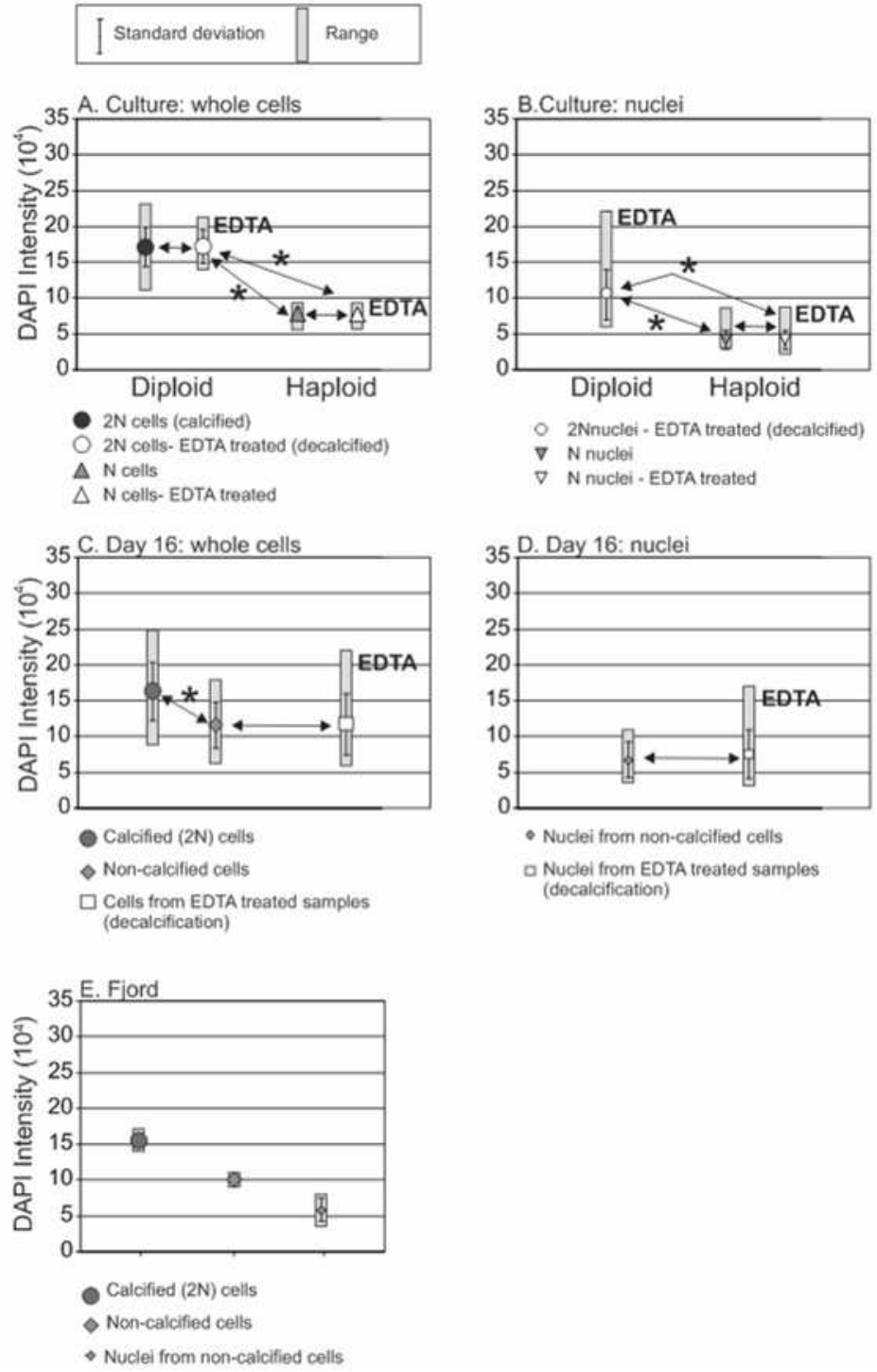


Figure 26. Quantitative analysis of DAPI stained *E. huxleyi* whole cells and nuclei from a cultured strain and from mesocosm samples. (A) Cultures whole cells: diploid cells (calcified) and EDTA treated (decalcified)) are present in the left; Haploid cells (normal and EDTA treated are present at right); (B) cultures nuclei: diploid nuclei (EDTA treated from decalcified cells) are present at left; Haploid nuclei from normal and EDTA treated samples are present at right; (C) Mesocosm samples from day 16 of whole cells (calcified and non-calcified) are presented at left; cells from EDTA teated samples are presented at right; (D) mesocosm samples from day 16 of nuclei: non-calcifed cells at presented at left; nuclei from cells treated with EDTA are presented at right; (E) combined normal and EDTA-treated samples from the Fjord (prior to the mesocosm experiment). Statistical comparitions are represented with arrows. * represent comparisons between two groups that are statistically different ($p<0.05$, Mann-Whitney).

In the fjord sample collected earlier, prior to the start of the mesocosm experiment, very few cells were present, which prevented statistical analyses (Fig. 26 E). However, all measurements were similar to those recorded on day 16, i.e. the calcified population had similar iDAPI values to the calcified population of day 16, the non-calcified population similar to the non-calcified population of day 16 and the nuclei were similar to the nuclei measured in day 16.

3.2.4. TEM analyses of the mesocosm samples

Samples from days 14, 15 and 17 were qualitatively analysed by TEM. Over this 4 days period, an increasing amount of *E. huxleyi* calcified cells (Fig. 27 A and B) infected with viruses (Fig. 27 C) was observed. Some of these cells had only a few coccoliths around them and the plasmalema was partially uncovered. Many free viral particles were also detected (Fig.27 D). Among the infected cells we found a single cell in the sample from bag 3 on day 14 that possessed organic scales typical of non-calcified haploid *E. huxleyi* (Fig. 27 E and F). Heterotrophic grazers were also observed with *E. huxleyi* cells infected with viruses inside cytoplasmic vacuoles.

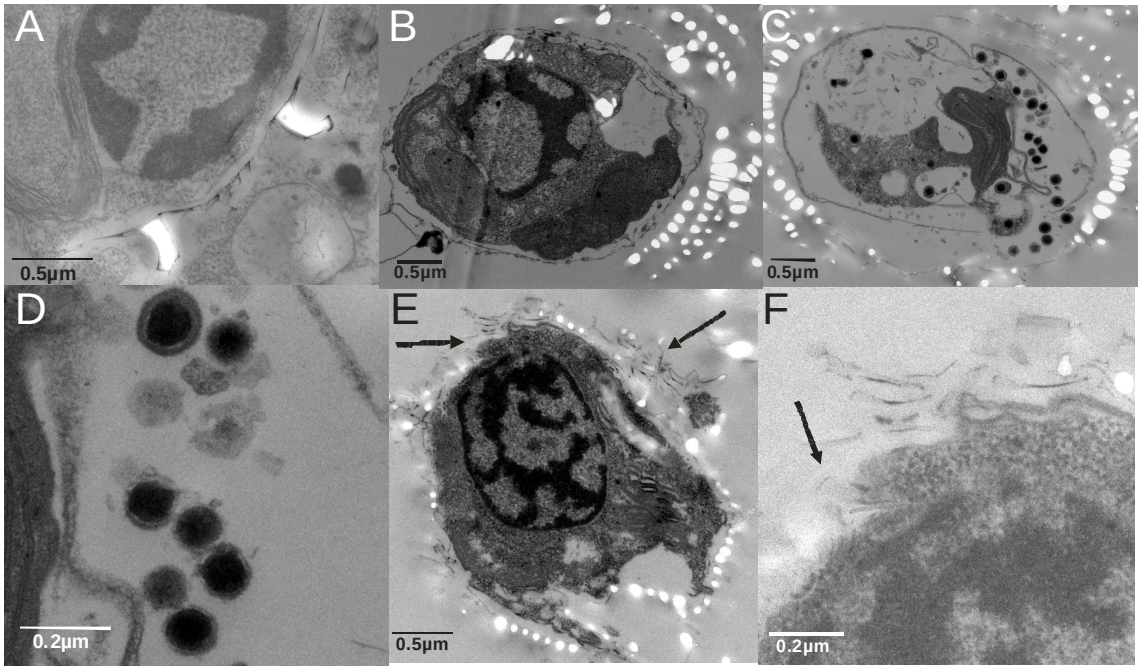


Figure 27. Transmission Electron Microscopy images from mesocosm samples.

(A) healthy *E. huxleyi* calcified diploid cell (a new coccolith in process of formation can be seen inside the cell); (B) *E. huxleyi* calcified diploid cell with the cell membrane partially exposed; (C) *E. huxleyi* calcified diploid cell infected with viruses; (D) Detail of a virus; (E) *E. huxleyi* haploid like-cell arrows show the scales that surround the cell); (F) Zoomed view of the scales of the haploid *E. huxleyi* like-cell.

3.2.5. *E. huxleyi* dynamics in other marine environments

Both calcified and non-calcified *E. huxleyi* cells were detected in all samples analysed using COD-FISH collected from both coastal and open ocean environments.

The coastal environments (Fig. 28 A and B) represented two well-mixed systems without major physic and chemical differences between the surface and the bottom water. In both cases, we detected a major calcified diploid *E. huxleyi* population (between 60 to 98% of the total *E. huxleyi* population) and a minor background non-calcified *E. huxleyi* population (between 2 to 40% of the total *E. huxleyi* population), with both populations exhibiting similar variations through time, i.e. increase in the density of calcified cells was accompained by increase in the density of the non-calcified cells. Empty *E. huxleyi* coccospheres were also detected and also followed the same dynamics. Note that samples from the SOMLIT-ASTAN station were analysed bi-weekly throughout the entire year 2007. In certain samples neither calcified nor non-calcified *E. huxleyi* cells were detected. Only the data concerning the period when *E. huxleyi* was present are shown. No *Gephyrocapsa* spp. cells were detected in either coastal locations and we therefore considered that all fluorescently labelled cells were *E. huxleyi*.

In the two stations of the oceanic environment off-shore Japan (Fig. 28 C), calcified cells of both *E. huxleyi* and *G. oceanica* were detected throughout the water column. Both species were thus considered together in our analysis. The two stations hydrographically divergent: a well mixed layer occurred at station B, whereas the water column was stratified at station F. Both calcified and non-calcifed *E. huxleyi* or *G. oceanica* cells were detected in the same range of depth distribution. Conversly to the coastal system presented above, here the cellular concentrations of both calcified and non-calcified cells displayed closer concentrations.

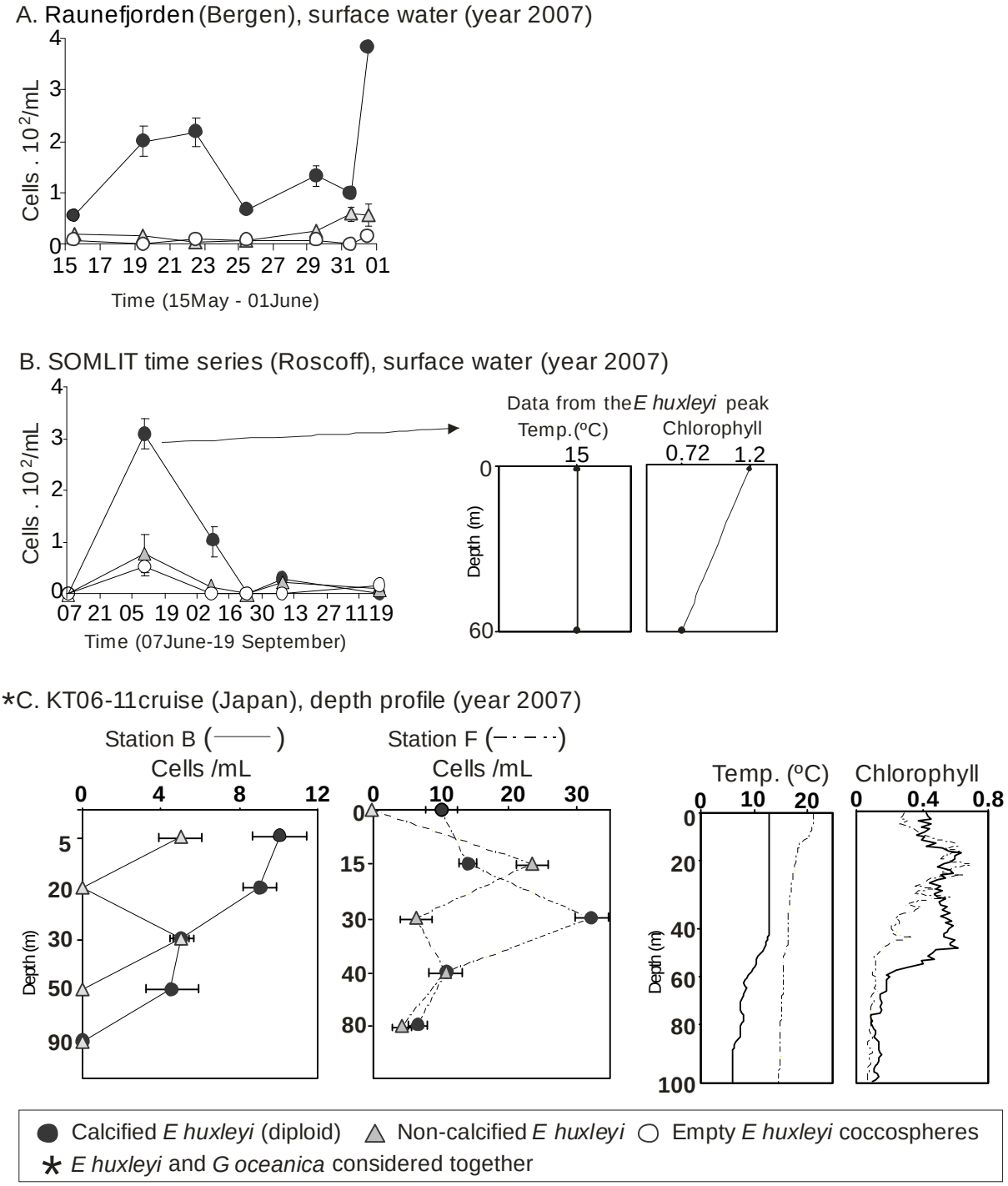


Figure 28. *E. huxleyi* dynamics in coastal and oceanic environments.

(A) Raunefjord time series (Bergen, Norway); (B) English Channel time series (Roscoff, France); (C) KT06-11 cruise (west Pacific, off-shore Japan) – in this situation *E. huxleyi* was considered together with *G. oceanica* (*).

4. Discussion

In the broad context of developing a holistic approach to understand the ecological dynamics of the dominant extant coccolithophore, *E. huxleyi*, we quantified the presence in

nature of calcified and non-calcified cells of this species using an innovative morpho-molecular method (COD-FISH) and a novel oligonucleotidic probe (EG28-03). These analyses were performed on samples collected over the time-course of a bloom in a mesocosm experiment and in other coastal and oceanic environments, where the overall cellular concentrations were much lower. From our sample set, we conclude that calcified cells (diploid) dominate *in situ E. huxleyi* populations, but non-calcifed *E. huxleyi* cells were also present at a relatively lower concentration and generally following the same population dynamics as the calcified phase cells.

4.1. Methodological remarks

Detection and quantification of calcified *E. huxleyi* cells (and other calcified coccolithophores) in samples collected from marine environments is normally performed by light or scanning electronic microscopy (Bown & Young 1998, Young et al. 2003) or by flow cytometry using their characteristically high side scatter as a distinguishing feature (Jacquet et al. 2002). In contrast, the non-calcified forms in the life cycle of *E. huxleyi*, due to their small size (ca. 4µm diameter) and lack of distinctive morphological features (i.e. a coccosphere), are almost impossible to distinguish from certain other prymnesiophytes using these methods. Fluorescent *in situ* hybridization (FISH) with specific genetic probes, classically designed for the 18S or 28S rDNA genes, coupled with cross-polarized microscopy that allow the visualization of the cells morphology and therefore recognize calcified and non-calcified cells, is a technical solution to this problem ((Frada et al. 2006), supplementary information 1). Our attempts to develop a FISH probe that would specifically hybridize with *E. huxleyi* were not successful because the 18S rDNA sequence of this species is identical to that of its sister-species *G. oceanica* (Edvardsen et al. 2000, Medlin & Zingone 2007), and the 28S rDNA sequences of these species differ in only a single base-pair mutation near the 3' end of the gene (Liu et al in prep.). Probes designed to this location never gave a positive signal and we therefore opted to design a probe targeting both species. This limitation could however be mitigated by checking by microscopy (SEM) for the presence of *G. oceanica* in the samples. *Gephyrocapsa* spp. were not present in the mesocosm, in the Raunefjord or in the English Channel samples. In these cases we assumed that fluorescently stained cells in COD-FISH analyses were only *E. huxleyi*. By contrast, both *E. huxleyi* and *G. oceanica* were detected in samples from Japanese waters, and both species were therefore considered together. In future studies aiming to exclusively target *E. huxleyi* with similar approaches in environments where

other Noelaerhabdaceae are known to be present, new probes will need to be designed targeting alternative, more divergent genetic regions. New systems that allow visualisation of cells targeted at the single gene level may offer the opportunity to do so (see (Zwirglmaier 2005) and references therein.).

Beyond the recognition of non-calcified cells in water samples, a main issue that remains unanswered with the COD-FISH approach, for the case of *E.huxleyi*, is the ploidy level of these cells. To address this issue, we developed an approach to quantitatively determine the DNA content of single cells of *E. huxleyi* and therefore evaluate the presence in natural samples of cells possessing similar or contrasting genome sizes, which could be indicative of the existence of different ploidy levels. This approach permitted successful descrimination of cultures of diploid and haploid cells. It proved necessary, however, to dissolve the coccoliths from the samples (through EDTA treatment) in order to clearly visualize the nuclei of the diploid calcified cells. This treatment was demonstrated to have no effect on the fluorescence intensity of DAPI stained nuclei. Following this tests made with cultured strains, we applied the protocol to mesocosm samples. Here, instead of pure clonal culture strains we dealt with an assemblage of *E. huxleyi* calcified and non-calcified cells. We further interpret these results in the next section.

4.2. *E. huxleyi* dynamics in a mesocosm bloom

The mesocosm experiment started before the formation of the natural bloom that typically occurs in Norwegian coastal waters at the end of spring (Heimdal et al. 1994). Several photosynthetic organisms, including calcified *E. huxleyi* (diploid) and other small haptophytes, were visualized by light microscopy in the fjord water used to fill the mesocosm enclosures. Both COD-FISH and DAPI-staining analyses revealed that some of these cells were non-calcified *E. huxleyi*, corresponding to 5-10% of the total *E. huxleyi* population. These non-calcifed cells were intact and apparently healthy, since they were well hybridized with the EG28-03 probe and the nucleus was clearly definied and visible after DAPI staining.

During the mesocosm experiment (Fig. 24), two nutrient regimes were tested: a phosphate limited condition (enclosure 2, ratio 75 nitrate (N): 1 phosphate (P)) and a non-limiting condition (enclosure 3, ratio 15 N: 1 P). In general, under both conditions, the *E. huxleyi* calcified population followed typical mesocosm dynamics (Bratbak et al. 1993, Castberg et al. 2001, Jacquet et al. 2002) with a lag period of 5 to 7 days, an exponential

phase followed by stationary phase of variable duration, and finally a population collapse. In bag 3, however, the exponential phase was longer and the cells attained twice the concentration compared to the P limited condition (bag 2). Also the stationary phase in bag 3 was practically non-existent, whereas in bag 2 the stationary phase lasted five days. These differences reflect the differential phosphate availability in the two bags (Wilson et al., in prep.). The collapse of both blooms occurred very rapidly after the massive proliferation of a population of specific viral particles. As demonstrated by Wilson et al. (in prep.), these viral particles were *Emiliania huxleyi* specific viruses (EhVs) and they were the main cause of the termination of the blooms (Wilson et al. in prep). In TEM micrographs from the terminal period of the bloom (days 15 to day 17), almost all calcified *E. huxleyi* cells observed were partially damaged, often lacking a defined nucleus and possessing viruses in the cytoplasm (Fig. 27 C).

The non-calcified *E. huxleyi* population followed similar dynamics to those described above for calcified cells, but as a minor background population that remained at relatively constant concentration in the first days of the experiment and then started progressively developing around day 11 (middle/end of the exponential phase of the calcified population) until days 14-15, with no major differences between the two nutrient regimes. At this point, we observed by light microscopy some groups of small flagellated haptophyte-like cells attached to coccoliths debris (Fig. 23 C and D) and in a subsequent TEM-micrograph we found a haptophyte cell possessing organic scales bound to the plasmalema (Fig. 27 E and F). These cells were probably haploid *E. huxleyi* cells produced by meiosis during this late stage of the bloom, maybe in response to the massive viral infection of diploid calcified cells. Supporting this hypothesis, a recent study demonstrated that haploid phase cells of *E. huxleyi* are not recognized and not infected by EhVs, and that their production, though meiosis, may be triggered in some diploid cells to escape viral infection and protect the species from extermination (Frada et al. 2008).

Subsequently, on day 16 we observed by COD-FISH a rapid increase of the non-calcified population, which coincided with the decline of the diploid calcified population. The abundance of non-calcified cells on this day allowed measurement of several DAPI stained cells and nuclei of both non-calcified and calcified cells (Fig. 26 C and D). In these analyses, we detected that the non-calcified population had on average lower iDAPI values compared to the calcified population, in contrast to the results obtained with cultures, and that their nuclei had a smaller range of sizes than the full *E. huxleyi* population (treated with EDTA). It is

therefore conceivable that part of this non-calcified population was composed of haploid cells possessing smaller genome sizes and thus iDAPI values. However, it likely also included a significant proportion of diploid non-calcified cells detected in the upper part of the range of whole cells and nuclei stained with DAPI. These non-calcifed cells that appeared suddenly on day 16 were thus probably mainly decaying diploid cells infected with viruses. This kind of cells is known to occur *in vitro* after viral infection and they rapidly vanish, usually 1 to 2 days later, probablydue to complete lysis of cells and/or bacterial degradation (Frada et al. 2008). Such a scenario could also explain the rapid decline of the non-calcified population in the mesocosm on day 17, one day after its sudden increase.

The same type of analysis can also be extrapolated to the iDAPI measurements perfomed in the fjord before the beginning of the mesocosm experiment (Fig. 26 E). Here we measured only samples without coccolith dissolution by EDTA, however, once again the non-calcified population was clearly smaller than the calcified population and the nuclei from this population had very low iDAPI values. It is therefore plausible that this population would be mainly composed by small haploid flagellated cells.

In this context, we hypothesise that the non-calcified population that we detected since the beginning in the fjord samples would be composed principally by haploid flagellated cells, that could be common in wild *E. huxleyi* populations. In a bloom situation this population would develop in parallel with the dominant calcified population, but would also be supplemented during the exponential growth phase of the diploid bloom by new haploid cells produced by meiosis in response to various stresses including viral attack. In this way it would constitue a seed population that could be used to colonise new virus-free areas and where it would produce through syngamy the next generation of diploid calcified cells.

Previous mesocosm studies have already documented the onset of a new population during the collapse of the bloom of calcified cells, characterised by a similar flow cytometric chlorophyll *a* fluorescence signal as the calcified phase, but lower side scatter values (Castberg et al. 2001, Jacquet et al. 2002). The authors suggested that these cells could represent other life cycle phases of *E. huxleyi*, i.e. diploid non-calcified or haploid flagellated cells. Our results support this hypothesis. However, our study stopped soon after the collapse of the diploid bloom and we could not follow the progression of the non-calcified population. New studies aiming to clarify the identity and further understand the role of *E. huxleyi* non-calcified phase, and particularly the role of the haploid flagellated phase, should survey the post-bloom period. Nevertheless, as we documented in this work, the haploid cells represent a

minor backgound population and the visualization of these cells constitutes a rare event. Other techniques, like for example quantitative-PCR (qPCR) employing haploid specific markers, could be used as complementary tools to quantify the haploid cells, but also monitor sexual (meiosis or syngamy) events during the development of blooms (Amato 2008).

4.3. Observations from the natural environment

In other marine environments we also detected both calcified and non-calcified *E. huxleyi* cells. However, due to sample limitations, we could not perform fluorescence intensity analyses of nuclei and TEM samples were not collected. Nevertheless, in both coastal systems (Raunefjord and ASTAN-SOMLIT time-series), it was clear that both the non-calcified and the calcified *E. huxleyi* cells were co-habiting in the same water masses throughout the studied periods. No seasonality, where different life cycle phases would peak at different periods of the year, were observed in these environments. In the west Pacific, however, the results were less conclusive since both *E. huxleyi* and *G.oceanica* (and probably other small *Gephyrocapsa* species) were analysed together. Nevertheless, both calcified and non-calcified forms were present together at all depths and there was no evidence of differential vertical distribution between these forms.

4.4. Ecological and evolutionary implications

Oceanic coccolithophores are considered to be K-selected species, preferring stratified water masses with low-nutrients (Margalef 1978, Young 1994). In some of these species a clear niche differentiation between life cycle phases has been demonstrated (e.g. *Helicosphaera carteri*, *Syracosphaera pulchra*, *Coronosphaera mediterranea*), the haploid (also calcified) phase typically being present in the surface layers and the diploid phase in deeper nutrient-rich waters (Cros et al. 2000, Cros 2002). Other species, like *Coccolithus pelagicus*, present a seasonality between life phases, i.e. when one phase dominates, the other is comparatively reduced and vice-versa (Okada & McIntyre 1977). It has been suggested that this partition strategy may allow species to widen their ecological niches, exploiting in 'two directions' the environmental opportunities (Valero et al. 1992, Cros 2002, Houdan et al. 2005, Houdan et al. 2006). *E. huxleyi*, by contrast, is considered to be the most r-selected coccolithophore, having high intrinsic growth rates, being highly predominant in fairly turbulent nutrient-rich environments and biogeographically widespread (Brand 1994, Young

1994). Moreover, the existence of a vertical stratification in its distribution is not always clear. In many well stratified oligotrophic environments, while the haploid and diploid life phase of K-selected coccolithophores appear clearly separated in depth, *E. huxleyi* appears spread throughout the entire water column (Okada & McIntyre 1977, Cros 2002). The mutual existence of both the calcified-diploid and the non-calcifed cells, including the flagellated-haploid cells that we detected in the mesocosm but that may also be common in wild populations, associated with a higher rate of life cycle turnover compared to other coccolithophore species, could explain this wide vertical distribution. While sinking, the non-motile diploid-calcifed cells would be replaced by swimming haploids, which could allow *E. huxleyi* to respond faster, in time and in space, to environmental changes, including massive viral infections produced during bloom formation (Frada et al. 2008). It could also permit more rapid access to new resources and/or more advantageous environments that would be preferentially exploitable by one or the other life cycle phase. However, some studies have reported a seasonal stratification of *E. huxleyi* calcified diploid populations in deeper waters (Cortes et al. 2001). These environments should be explored in the future in order to investigate whether non-calcified cells occupy different (shallower) water layers during these seasons.

More detailed knowledge of *E. huxleyi* life cycle dynamics would provide insights into the evolution and diversification of this important species. It is generally thought that sexual populations, through genetic recombination, may evolve faster than asexual populations, allowing them to adapt more rapidly to changing environments (including aboitic and biotic challenges) (Maynard Smith & Szathmáry 1999). High recombination rates together with the possible wide geographic dispersal promoted by planktonic life in the oceans (Brand 1994, Young 1994) could instigate population divergence, local adaptations and speciation. Indeed, presently, at least four morphotypes are recognized within the *E. huxleyi* species (Young et al. 2003) and the existence of physiological differences between strains collected from diverse marine ecosystems has been demonstrated (e.g. (Brand 1994, Young 1994, Paasche 2001) Paasche 2001). This phenotypic variability suggests, as has been recognized for other coccolithophores species (Saez et al. 2003), that *E. huxleyi* is composed of different cyptic species, that would be locally adapted to specific ecological niches. An elevated sexual (life cycle) turnover, that has been suggested to occur in *E. huxleyi* (Iglesias-Rodriguez et al. 2006), would further enhance this phenomenon and could therefore be the basis for the profusion of this young species throughout the oceans since its apperence only 270,000 years ago (Thierstein et al. 1977).

4.5. Concluding remarks

Knowledge of the ecology of *E. huxleyi* as a whole, comprising both life cycles phases, is still incomplete. Other environments, including natural blooms and temperate waters presenting a clear seasonality and where *E. huxleyi* is present throughout the year, and other technical approaches like qPCR should be explored in the future in order to test the conclusions presented above. Nevertheless, it is now clear that calcified and non-calcified *E. huxleyi* cells dwell together in the plankton and that both contribute to the ecological impact and evolution of *E. huxleyi*. Moreover, there are major biogeochemical differences between these life cycle phases. The diploid phase is calcified, whereas the other phases, diploid or haploid, are non-calcified. Their specific contributions to the oceanic carbon budgets is thus completely different. In addition, their responses to predicted environmental changes, such as ocean acidification and temperature rises (Westbroek et al. 1993, Rost & Riebesell 2004, Iglesias-Rodriguez et al. 2008), will also probably be different. The detection, quantification and relative importance of non-calcified cells should therefore be included in oceanographic models and taken into account when referring to *E. huxleyi*.

5. Acknowledgements

We deeply acknowledge all mescosm 2008 community, namely A. Aadnesen for her overall help. We would like to thank also to: F.Jalabert and the ‘service mer’ from the Station Biologique de Roscoff, for the samples and physico-chemical data from the SOMLIT-ASTAN point; Y. Okazaki and the TANSEI MARU crue (JAMSTEC, Japan); J.Suriman and S.Le Panse and the ‘Platform d’imagerie’ of the Station Biologique de Roscoff. This work was supported by: a Ph.D. fellowship from the “*Fundação Para a Ciência e para a Tecnologia*” (Portugal) and a Algal node student scholarship, ('Marine Genomics Europe' - European Commission contract No. GOCE-CT-2004-505403), both attributed to MF; and an ATIP grant awarded to CdV by the “*Centre National de la Recherche Scientifique*”, France. This work is part of the pluridisciplinary project *BOOM* - Biodiversity of Open Oceans Microalcifiers funded by the French “Agence National de la Recherche”, grant ANR-05-BIODIV-004.

6.References

Amann RI, Ludwig W, Schleifer K-H (1995) Phylogenetic Identification and In Situ Detection of Individual Microbial Cells Without Cultivation. Microbiological Reviews 59:143-169

Amato A (2008) The sexual cycle of the diatom *Pseudo-Nitzschia*: from morphology through biology to genes. The Open University

Billard C (1994) Life cycles. In: Green JC, Leadbeater BSC (eds) The Haptophyta Algae, Vol 51. Clarendon Press, Oxford, p 167-186

Bown P, Young J (1998) Calcareous nannofossil biostratigraphy, Vol. Kluwer Academic Publishers, Cambridge

Brand LE (1994) Physiological ecology of marine coccolithophores. In: Winter A, Siesser WG (eds) Coccolithophores. Cambridge University Press, Cambridge

Bratbak G, Egge J, Heldal M (1993) Viral mortality of the marine alga *Emiliania huxelyi (*Haptophyceae) and the termination of the algal bloom. Marine Ecology Progess Series 93:39-48

Brown CW, Yoder JA (1994) Distribution pattern of coccolithophorid blooms in the Western North Atlantic Ocean. Continental Shelf Research 14:175-198

Campbell L, Shapiro LP, Haugen E (1994) Immunochemical Characterization of Eukaryotic Ultraplankton from the Atlantic and Pacific Oceans. Journal of Plankton Research 16:35-51

Castberg T, Larsen A, Sandaa RA, Brussard CPD, Egge JK, Heldal M, Thyrhaug R, van Hannen EJ, Bratbak G (2001) Microbial population dynamics and diversity during a bloom of the marine coccolithophorid Emiliania huxleyi (Haptophyta). Marine Ecology Progress Series 221:39-46

Cortes MY, Bollmann J, Thierstein HR (2001) Coccolithophore ecology at the HOT station ALOHA, Hawaii. Deep-Sea Research Part Ii-Topical Studies in Oceanography 48:1957-1981

Cros L (2002) Planktonic Coccolithophores of the NW Mediterranean., Universitat de Barcelona

Cros L, Kleijne A, Zeltner A, Billard C, Young JR (2000) New examples of holococcolith-heterococcolith combination coccospheres and their implications for coccolithophorid biology. Marine Micropaleontology 39:1-34

Edvardsen B, Eikrem W, Green JC, Andersen RA, Moon-van der Staay SY, Medlin L, K. (2000) Phylogentic reconstruction of the Haptophyta inferred from the 18S ribosomal DNA sequences and available morphological data. Phycologia 39:19-35

Egge JK, Heimdal BR (1994) Blooms of Phytoplankton Including Emiliania-Huxleyi (Haptophyta) - Effects of Nutrient Supply in Different N-P Ratios. Sarsia 79:333-348

Frada M, Not F, Probert I, de Vargas C (2006) $CaCO_3$ optical detection with fluorescent in situ hybridization: a new method to identify and quantify calcifying microorganisms from the oceans. Journal of Phycology 42:1162-1169

Frada M, Probert I, Allen MJ, Wilson WH, De Vargas C (2008) The “Cheshire Cat” escape strategy of the coccolithophore *Emiliania huxleyi* in response to viral infection Proceedings of the National Academy of Sciences USA 105:15944–15949

Green JC, Course PA, Tarran GA (1996) The life-cycle of *Emiliania huxleyi*: A brief review and a study of relative ploidy levels analysed by flow cytometry. J Mar Syst 9:33-44

Heimdal BR, Egge JK, Veldhuis MJW, Westbroek P (1994) The 1992 norwegian *Emiliania huxleyi* experiment - an overview. Sarsia 79:285-290

Holligan PM, Fernandez E, Aiken J, Balch WM, Boyd P, Burkill PH, Finch M, Groom SB, Malin G, Muller K, Purdie DA, Robinson C, Trees CC, Turner SM, Van der Wal P

(1993) A biogeochemical study of the coccolithophore Emiliania huxleyi, in the north atlantic. Global Biogeochemical Cycles 7:879-900

Houdan A, Probert I, Van Lenning K, Lefebvre S (2005) Comparison of photosynthetic responses in diploid and haploid life-cycle phases of *Emiliania huxleyi* (Prymnesiophyceae). Marine Ecology Progress Series 292:139-146

Houdan A, Probert I, Zatylny C, Véron B, Billard C (2006) Ecology of oceanic coccolithophores. I. nutritional preferences of the two stages in the life cycle of *Coccolithus braarudii* and *Calcidiscus leptoporus*. Aquatic Microbial Ecology 44:291-301

Iglesias-Rodriguez MD, Halloran PR, Rickaby REM, Hall IR, Colmenero-Hidalgo E, Gittins JR, Green DRH, Tyrrell T, Gibbs SJ, von Dassow P, Rehm E, Armbrust EV, Boessenkool KP (2008) Phytoplankton calcification in a high-CO_2 world. Science 320:336-340

Iglesias-Rodriguez MD, Schofield OM, Batley J, Medlin LK, Hayes PK (2006) Intraspecific genetic diversity in the marine coccolithophore *Emiliania huxleyi* (Prymnesiophyceae): The use of microsatellite analysis in marine phytoplankton population studies. Journal of Phycology 42:526-536

Jacquet S, Heldal M, Iglesias-Rodriguez D, Larsen A, Wilson W, Bratbak G (2002) Flow cytometric analysis of an *Emiliana huxleyi* bloom terminated by viral infection. Aquatic microbial ecology 27:111-124

Klaveness D (1972) Coccolithus huxleyi (Lohm.) Kamptn. II. The flagellate cell. aberrant cell types, vegetative propagation and life cycles. British Phycological Journal 7:309-318

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, Konig A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH (2004) ARB: a software environment for sequence data. Nucleic Acids Research 32:1363-1371

Malin G, Steinke M (2004) Dimethyl Sulfide Production: What is the Contribution of the Coccolithophores? In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Margalef R (1978) Life-forms of phytoplankton as survival alternatives in an unstable environment. Oceanologica Acta 1:493-509

Martinez JM, Schroeder DC, Larsen A, Bratbak G, Wilson WH (2007) Molecular dynamics of *Emiliania huxleyi* and cooccurring viruses during two separate mesocosm studies. Appl. Environ. Microbiol. 73:554-562

Maynard Smith J, Szathmáry E (1999) The Origins of life, from the birth of life to the origin of language, Vol. Oxford University Press Inc, New York

McIntyre A, Bé AW (1967) Modern coccolithophoridae of the atlantic ocean- I. placoliths and cyrtoliths. Deep-sea research 4:561-597

Medlin LK, Zingone A (2007) A taxonomic review of the genus Phaeocystis. Biogeochemistry

Nanninga HJ, Tyrrell T (1996) The importance of light for the formation of algal blooms by *Emiliania huxleyi*. Marine Ecology Progess Series 136:195-203

Okada H, McIntyre A (1977) Modern coccolithophores of the Pacific and North Atlantic Oceans. Micropaleontology 23:1-55

Paasche E (2001) A review of the coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae), with particular reference to growth, coccolith formation, and calcificationphotosynthesis interactions. Phycologia 40:503-529

Rost B, Riebesell U (2004) Coccolithophores calcification and the biological pump: reponse to environmental changes. In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Saez AG, Probert I, Geisen M, Quinn P, Young JR, Medlin LK (2003) Pseudo-cryptic speciation in coccolithophores. Proceedings of the National Academy of Sciences 100:7163-7168

Simon N, Campbell L, Erla Örnlfsdttir E, Groben R, Guillou L, Lange M, Medlin LK (2000) Oligonucleotide probes for the identification of three algal groups by dot blot and fluorescent whole-cell hybridization. The Journal of Eukaryotic Microbiology 47:76-84

Thierstein H, Geitzenauer K, Molfino B, Shakleton N (1977) Global synchroneity of late Quarternary coccolith datum levels: Validation by oxygen isotopes. Geology 5:400-4004

Tyrrell T, Merico A (2004) *Emiliania huxleyi*: bloom observation and the conditions that induce them. In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Urdea MS, Warner BD, Running JA, Stempien M, Clyne J, Horn T (1988) A comparison of non-radioisotopic hybridization assay methods using fluorescent, chemiluminescent and enzyme labeled synthetic oligodeoxyribonucleotide probes. Nucleic Acid Research 16:4937-4956

Valero M, Richerd S, Perrot V (1992) Evolution of alternation of haploid and diploid phases in life cycles. Trends in Ecology and Evolution 7:25-29

Westbroek P, Brown CW, Van Bleijswijk J, Brwonlee C, Brummer GJ, Cote M, Egge J, Fernandez E, Jordan R, Knappersbusch M, Steffels J, Veldhuis M, Van der Wal P, Young J (1993) A model system approach to biological climate forcing: the example of Emiliania huxleyi. Global Planet Change 8:27-46

Wilson WH, Tarran GA, Schroeder D, Cox M, Oke J, Malin G (2002) Isolation of viruses responsible for the demise of an *Emiliania huxleyi* bloom in the English Channel. J. Mar. Biol. Ass. U.K. 82:369-377

Young G (1994) Functions of coccoliths. In: Winter A, Siesser WG (eds) Coccolithophores. Cambridge University Press, Cambridge, p 63-82

Young J, Geisen M, Cros L, Kleijne A, Sprengel C, Probert I, Østergaard J (2003) A guide to extant coccolithophore taxonomy. Journal of Nannoplankton Research:125 pp.

Zwirglmaier K (2005) Fluorescence *in situ* hybridisation (FISH) - the next generation. Fems Microbiology Letters 246:151-158

Supplementary information 5

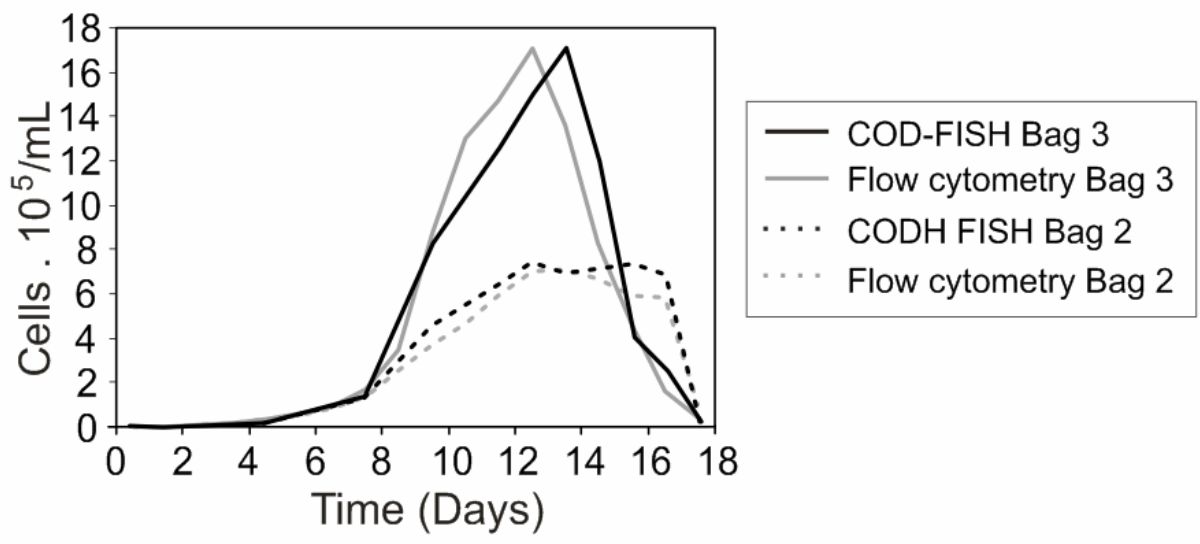


Figure legend. COD-FISH and flow cytometric survey of *E. huxleyi* calcified cells during the mesocosm experiment (enclosure 2 and 3).

PART III. *OTHER STUDIES ON HAPTOPHYTES LIFE CYCLES DIVERSITY AND EVOLUTION*

CHAPTER 4. First observations of heterococcolithophore-holococcolithophore life cycle combinations in the family Pontosphaeraceae (Calcihaptophycideae, Haptophyta)

First observations of heterococcolithophore-holococcolithophore life cycle combinations in the family Pontosphaeraceae (Calcihaptophycideae, Haptophyta)

Miguel Frada[1,2]*, Isabella Percopo[3], Jeremy Young[4] , Adriana Zingone[3], Colomban de Vargas[1] and Ian Probert[1]

[1]CNRS & Université Pierre et Marie Curie (UMR 7144), Equipe EPPO - Evolution du Plancton et Paléo-Océans, Station Biologique, 29682 Roscoff, France

[2]Centro de Geologia, Faculdade de Ciências, Universidade de Lisboa, Edifício C6, Campo Grande, 1749-016 Lisboa, Portugal

[3]Stazione Zoologica 'A. Dohrn', Villa Comunale, 80121-Naples, Italy

[4]Paleontology Department, Natural History Museum, Cromwell Road, London SW75BD, UK

*corresponding author:
Miguel Frada
Station Biologique
Equipe EPPO - Evolution du Plancton et Paléo-Océans (UMR 7144)
Place Georges Teissier, Roscoff (France)
Phone : 33 (0) 2-98-29-25-28
email : frada@sb-roscoff.fr

In Press in : **Marine Micropaleontology**

⌬ the structure of the manuscript submitted to Marine Micropaleontology was maintained

Abstract

Coccolithophores (Calcihaptophycideae, Haptophyta) are characterised by a heteromorphic life cycle with alternating haploid and diploid generations. Typically, diploid cells produce heterococcoliths, whereas haploid cells produce holococcoliths. An important source of evidence for coccolithophore life cycles comes from observations of combination coccospheres, which are cells bearing both types of coccoliths and thus interpreted as capturing the transition from haploid to diploid phase or vice-versa. Such observations are rare and many heterococcolith and holococcolith-bearing taxa remain non-associated to a life cycle phase counter-part. Information on life cycles is even completely missing for entire lineages. Here we report the first observations of heterococcolithophore–holococcolithophore combinations in the family Pontosphaeraceae, consisting of the associations of the morpho-species *Scyphosphaera apsteinii* with *Syracolithus schilleri* and of *Pontosphaera japonica* with an undescribed *Syracolithus*-like holococcolithophore. These new observations provide clear evidence that the Pontosphaeraceae have dimorphic haplo-diploid life cycles, further supporting the hypothesis that this life cycle pattern is ubiquitous among calcihaptophytes. Moreover, our results highlight a high degree of morphological convergence between holococcolithophores belonging to different lineages, since *Syracolithus*-like holococcolithophores are also present in the life cycle of species within the Helicosphaeraceae and Calcidiscaceae, the latter family being phylogenetically distant from the Pontosphaeraceae. These morphological resemblances may provide evidence for: (1) morpho-structural constraints on holococcolith construction, and/or (2) ecological convergence of the haploid phases of different coccolithophores.

Keywords

Coccolithophores; Pontosphaeraceae; Life cycles; Dimorphism; Convergent evolution

1. Introduction

Prymnesiophytes, including coccolithophores, are characterised by a heteromorphic life cycle with alternating haploid and diploid generations produced via the sexual processes of meiosis and syngamy respectively (Billard 1994, Houdan et al. 2004). Both phases are capable of mitotic reproduction and hence of producing autonomous populations. The broad phylogenetic distribution of haplo-diploid life cycles in prymnesiophytes suggests that this is a synapomorphic state for the group, and possibly for the haptophytes as a whole (Young et al. 2005, de Vargas et al. 2007). The ability to grow vegetatively under two phases expressing distinct phenotypes should therefore be of primary evolutionary significance. It may be a strategy for a species to exploit a wider range of ecological conditions (Valero et al. 1992, Cros et al. 2000), and/or to rapidly escape negative selection pressures exerted on one stage, such as grazing, parasitic attack or viral infections (Frada et al. 2008), or abrupt environmental changes (Noel et al. 2004).

Typically in the coccolithophore, clade (the Calcihaptophycideae -(de Vargas et al. 2007)), the diploid phase produces heterococcoliths (calcareous scales composed of radial arrays of complex crystal units of variable shape) while the haploid phase either produces holococcoliths (calcareous scales composed of associations of numerous minute identical euhedral crystallites) (Billard 1994, Young et al. 2005) or is non-calcified as in the ubiquitous species *Emiliania huxleyi* and other members of the Noelaerhabdaceae ((Green et al. 1996), our unpublished data). The best evidence for the existence of haplo-diploid life cycles in calcihaptophytes comes from clonal culture observations that provide unambiguous proof of the association of different phenotypes and allow determination of their ploidy levels (Houdan et al. 2004). However, only a small subset of total coccolithophore diversity has been maintained in culture and direct observations of life cycle changes in culture remain infrequent since the triggers to induce life-cycle changes have not been identified. For example, *Scyphosphaera apsteinii* has been maintained in culture in our collection for 8 years without ever undergoing a life-cycle transition. Combination coccospheres, bearing both hetero- and holococcoliths, therefore provide an invaluable additional source of evidence. Sporadically recorded in natural samples from various oceanic regions (Thomsen et al. 1991, Cros et al. 2000, Cortés & Bollman 2002, Geisen et al. 2002), combination coccospheres are interpreted as cells captured shortly after a life-cycle transition so that they bear coccoliths from both the previous and the new life-cycle stages.

Traditionally, coccolithophore taxonomy has been almost exclusively based on morphological characters of the coccoliths covering the cells, including crystallographic orientation of the component crystal units (Bown & Young 1998, Young et al. 1999). A significant consequence of this is that the radically different morphological entities within the coccolithophores life cycle have been classified as separate species in different families. To date less than 30% of holococcolithophore (HOL) morphotypes have been associated with their heterococcolithophore (HET) counterpart. From these few cases, the difficulty of predicting life cycle associations based on holococcolith morphology is already clear. Members of the holococcolithophore morphological genus *Crystallolithus* (Gaarder and Markali 1956) have been shown to be associated with heterococcolithophore species from two distinct (but relatively closely related) families, the Calcidiscaceae and the Coccolithaceae. A more striking example comes from the fact that holococcolithophore morphotypes from the genus *Syracolithus* Deflandre 1952 are associated with heterococcolithophore species within the Calcidiscaceae and Helicosphaeraceae, two families whose most recent common ancestor was in the early Mesozoic (de Vargas et al. 2007). *Syracolithus* holococcoliths show diverse morphologies and although some are already associated with a heterococcolithophore form (*Syracolithus quadriperforatus/Calcidiscus quadriperforatus*; *Syracolithus catilliferus* and *Syracolithus confusus/Helicosphaera carteri*), most species within the genus (*Syracolithus schilleri*, *S. dalmaticus*, *S. ponticuliferus*, *S. bicorium*, *S.* sp. type A, *S.* sp. type B) have unknown heterococcolith counterpart (Kleijne 1991, Young et al. 2003).

Here, we report the first two observations of life cycle associations within the calcihaptophyte family Pontosphaeraceae Lemmerman 1908. The presumably diploid stage of members of this family produce large, heavily calcified heterococcoliths (muroliths) with a central area displaying a variable number of perforations. No evidence of a sexual life cycle has previously been reported in the Pontosphaeraceae (Young et al. 2003, Young et al. 2005). This report is based on the observation of two combination coccospheres from natural samples, one associating the heterococcolithophore *Scyphosphaera apsteinii* Lohmann 1902 and the holococcolithophore *Syracolithus schilleri* (Kampter 1927) Loeblich & Tappan 1963 and the other the heterococcolithophore *Pontosphaera japonica* (Takayama 1967) Nishida 1971 and a new holococcolithophore, morphologically similar to, yet distinct from, both *Syracolithus confusus* and *S. schilleri* (Young et al. 2003). We discuss the implications of these new observations for the taxonomy of the genus *Syracolithus* and, in a wider context, for the evolution of the sexual life cycles in coccolithophores.

2. Material and Methods

The seawater sample containing the *S. apsteinii – Syracolithus schilleri* combination coccosphere was collected with a 5µm mesh plankton net from surface water in the North Atlantic "38 degrees 18'N 30 degrees 04'W" during a trans-Atlantic cruise (Atlantic Meridional Transect 16) on the 23rd June 2005 (water temperature 19.3 ºC, salinity 36.2 PSU; for further details see (Robinson et al. 2006, Poulton et al. 2007)). The sample was fixed for 1h at 4ºC with paraformaldehyde (pH 8) at a final concentration of 1%. Subsequently it was filtered onto a 0.2µm anodisc filter (Whatman, Maidstone, UK) and dried at room temperature. Later, the filter was embedded in immersion oil (Olympus 04 Japan, Olympus Optical Co., Tokyo, Japan), mounted between a glass slide and a cover slip and observed in cross-polarized light and in Nomarski Interference Contrast with an Olympus BX51 microscope (Olympus Optical Co. Ltd., Tokyo, Japan). For subsequent scanning electron microscopy the filter was retrieved from the slide, rinsed with TNT buffer (10mM Na_2CO_3, 5mM Tween 20, 0,074% NaCl, 20mM Tris pH 9), and dried at 55ºC. The filter was then mounted on an aluminium stub, sputter coated with Au-Ir and examined in a Phillips XL 30 FEG scanning electron microscope.

The seawater sample containing the *P. japonica* HET/ HOL combination coccosphere was collected from surface water with a 5µm mesh size plankton net at an offshore station in the Tyrrhenian Sea "39 degrees 30' N 13 degrees 30' E" on the 24th November 2006. The sample was fixed with formaldeyde at a final concentration of 2%. Observations were made with a Zeiss Axiovert microscope equipped with a Zeiss Axiocam digital camera. For SEM observations, 2ml of sample were filtered onto a 0.8µm Isopore filter (Millipore, Ireland), rinsed with tap water and dried at room temperature. The filter was mounted on an aluminium stub, sputtered with platinum (Pt) and observed with a JEOL 6700F scanning electron microscope. For determination of the crystallographic orientation of the holococcolith crystals an aliquot of the sample was filtered onto a 1µm pore size cellulose acetate filter, permanently mounted using Norland Optical Adhesive (NOA74) and examined under cross-polarised illumination using a Zeiss Axioplan photomicroscope. The methodology for this type of analysis is described in e.g. (Moshkovitz & Osmond 1989). Morphometric analyses (length, width and number of pores) of holococcoliths of both species were performed on SEM images.

3. Results

3.1. Scyphosphaera apsteinii - Syracolithus schilleri

Scyphosphaera apsteinii is a highly distinctive coccolithophore with a dimorphic coccosphere consisting of flat, disk-like muroliths and elevated bowl-like equatorial coccoliths, known as lopadoliths (Young et al. 2003, Young 2008). The *S. apsteinii – Syracolithus schilleri* combination coccosphere presented here was initially detected by light microscopy, and then transferred to a scanning electron microscope to confirm the initial observations (Fig. 29). The combination coccosphere consisted of 12 heterococcoliths and ~30 holococcoliths. The heterococcoliths included eight disk-like body coccoliths and two elevated bowl-like equatorial lopadoliths. The coccosphere was collapsed, therefore both the heterococcoliths and holococcoliths occurred in two layers, with a lower layer arranged with the distal face downwards and an upper layer with the distal face upwards (Fig. 29A). Most holococcoliths were found in the centre-right of the coccosphere and most heterococcoliths around its periphery. The two kinds of coccoliths overlapped, with examples of both heterococcoliths overlying holococcoliths and vice versa. The holococcoliths from this specimen were 3.3-3.7 µm in length, 2.35-2.5 µm wide and thickness varied between 0.85-1.34 µm. They have 10 to 14 (very rarely 15) pores (average = 11.4, n = 26). The central area of the laminolith holococcoliths is raised and shows a conical central protuberance (Fig. 29 and Fig. 31A). In cross-polarized microscopy, the holococcoliths are characterised by a bright outermost wall forming a tube around a darker inner part clearly subdivided by internal septae (Fig. 29C and Fig. 31A). This appearance reflects the fact that *S. schilleri* holococcoliths are formed of an outer wall of crystallites with radially directed sub-horizontal *c*-axes and an inner mass of several layers of crystallites with vertically directed *c*-axes (Young et al. 2003).

The seawater sample contained an abundant coccolithophore assemblage including heterococcolith-bearing cells of *Emiliania huxleyi*, *Helicosphaera carteri*, *Calcidiscus leptoporus, Discosphaera tubifera, Umbellosphaera tenuis*, and rare *Scyphosphaera apsteinii* HET. Holococcolith bearing cells were also common including *Helicosphaera carteri* HOL (previously known as *Syracolithus catilliferus*), *Syracosphaera anthos* HOL (previously known as *Periphyllophora mirabilis*) and several other holococcolithophore species. However, no other specimens of *Syracolithus schilleri* were found.

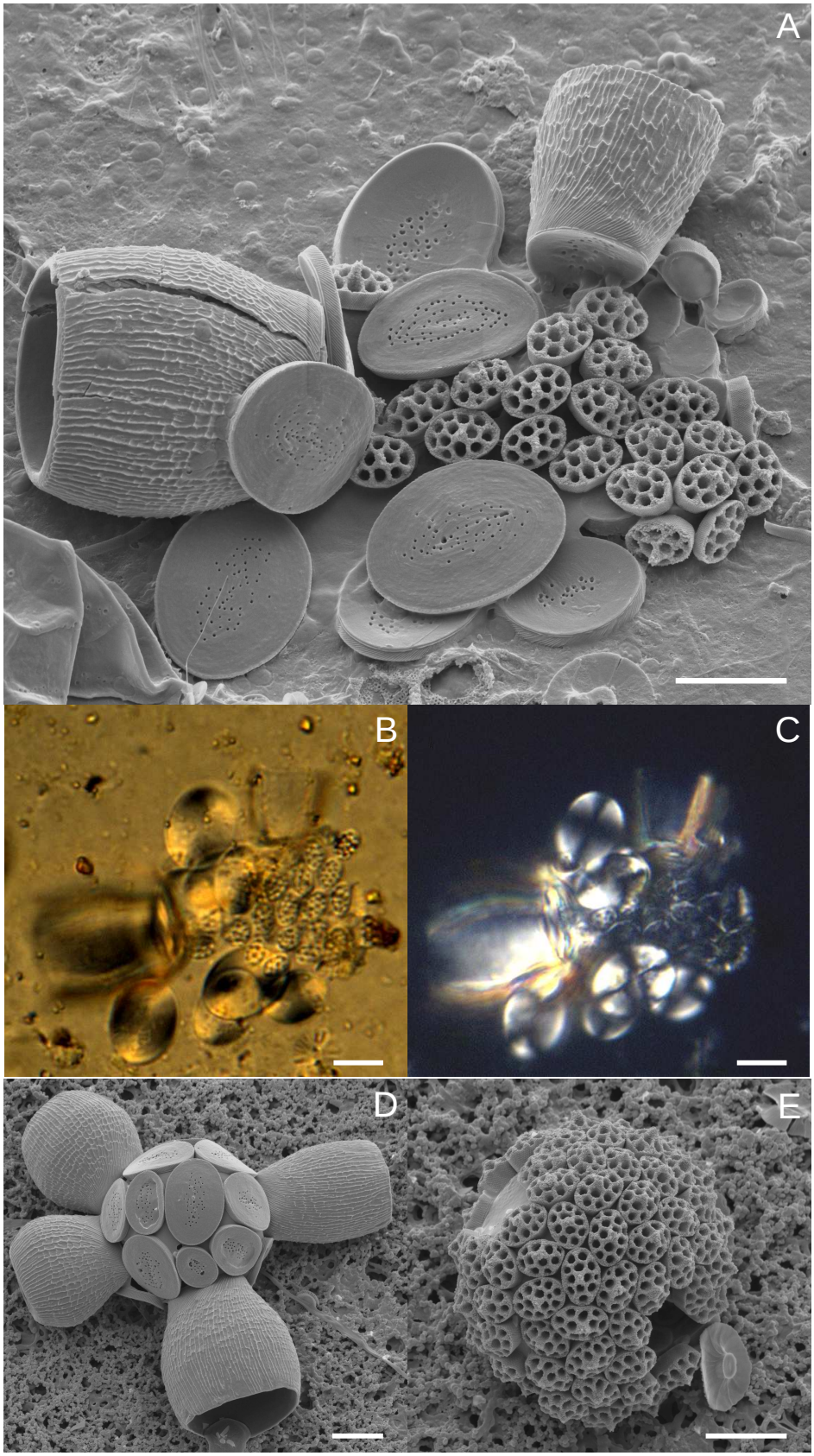


Figure 29. *Scyphosphaera apsteinii – Syracolithus schilleri* combination coccosphere. (A) SEM image of the whole combination coccosphere; (B) Nomarski Interference Contrast and (D) cross polarization microscopy images of same specimen; SEM images of (E) *S. apsteinii* and (F) *Syracolithus schilleri* coccospheres collected at the Hawaiian HOTS station. (scale bar = 5 µm).

3.2. Pontosphaera japonica HET/HOL

Pontosphaera japonica has a monomorphic coccosphere composed of muroliths with numerous very small pores (ca. 0.05µm diameter) in the central area (Young et al. 2003). The new *P. japonica* life cycle association was observed by both light and scanning electron microscopy (Fig. 30). A total of nine combination coccospheres were observed (five in LM and four in SEM). Cross-polarized observations and analyses of the specimens were carried out *a posteriori* from preserved samples (Fig. 30 E and Fig. 31 B).

The combination coccospheres were perfectly preserved in most of the specimens observed. They consisted of 5-12 heterococcoliths and of 16-36 holococcoliths. The length, width and thickness of the holococcoliths were 3.55 – 4.01 µm, 2.67 – 2.83 µm, and 0.83-0.96 µm, respectively. The distal surface of the coccoliths has a variable number of pores (4-11, average = 7.2, n = 27) of different size (0.3-0.8 µm diameter) (Fig. 30). The central area of the laminoliths is raised and generally possesses one or two protuberances, with their tip surrounded by concentric ridges. The coccoliths lacked a distinct outer wall and hence the horizontal laminar arrangement of crystallites is directly exposed to the exterior, appearing as 8-10 parallel layers of crystals (Fig. 31 B).

In cross-polarised light, the holococcoliths were dark in planar view in all orientations indicating that the calcite crystallites are aligned with their crystallographic c-axes vertical. (Fig. 30 E and Fig. 31 B).

Two coccospheres composed exclusively of holococcoliths were observed by LM and two specimens of *Pontosphaera japonica* HET were detected. The sample also contained a diverse and abundant coccolithophore population consisting of 24 species, including both heterococcolith-bearing and holococcolith-bearing cells. The dominant heterococcolithophores were *Syracosphaera pulchra*, *Scyphosphaera apsteinii*, *Coronosphaera mediterranea*, *Ceratolithus cristatus* and *Helicosphaera carteri*. Among the holococcolithophores, the most frequent were *Syracosphaera pulchra* HOL *pirus* type (previously known as *Calyptrosphaera pirus*), *Coronosphaera mediterranea* HOL *wettsteinii*-type (previously known as *Calyptrolithina wettsteinii*), *Calyptrolithina divergens* var. *tuberosa* and *Calyptrolithophora gracillima*. Several species belonging to the genus *Pontosphaera* (*P. syracusana*, *P. discopora* and *P. multipora*) were present, although these were very rare. Combination coccospheres of *Syracosphaera pulchra* and *S. pulchra* HOL *pirus* type were also detected. The low abundance of smaller coccolithophores, *Emiliania* and *Gephyrocapsa*, reflected the relatively large mesh size (5 µm) of the plankton net used to collect this sample.

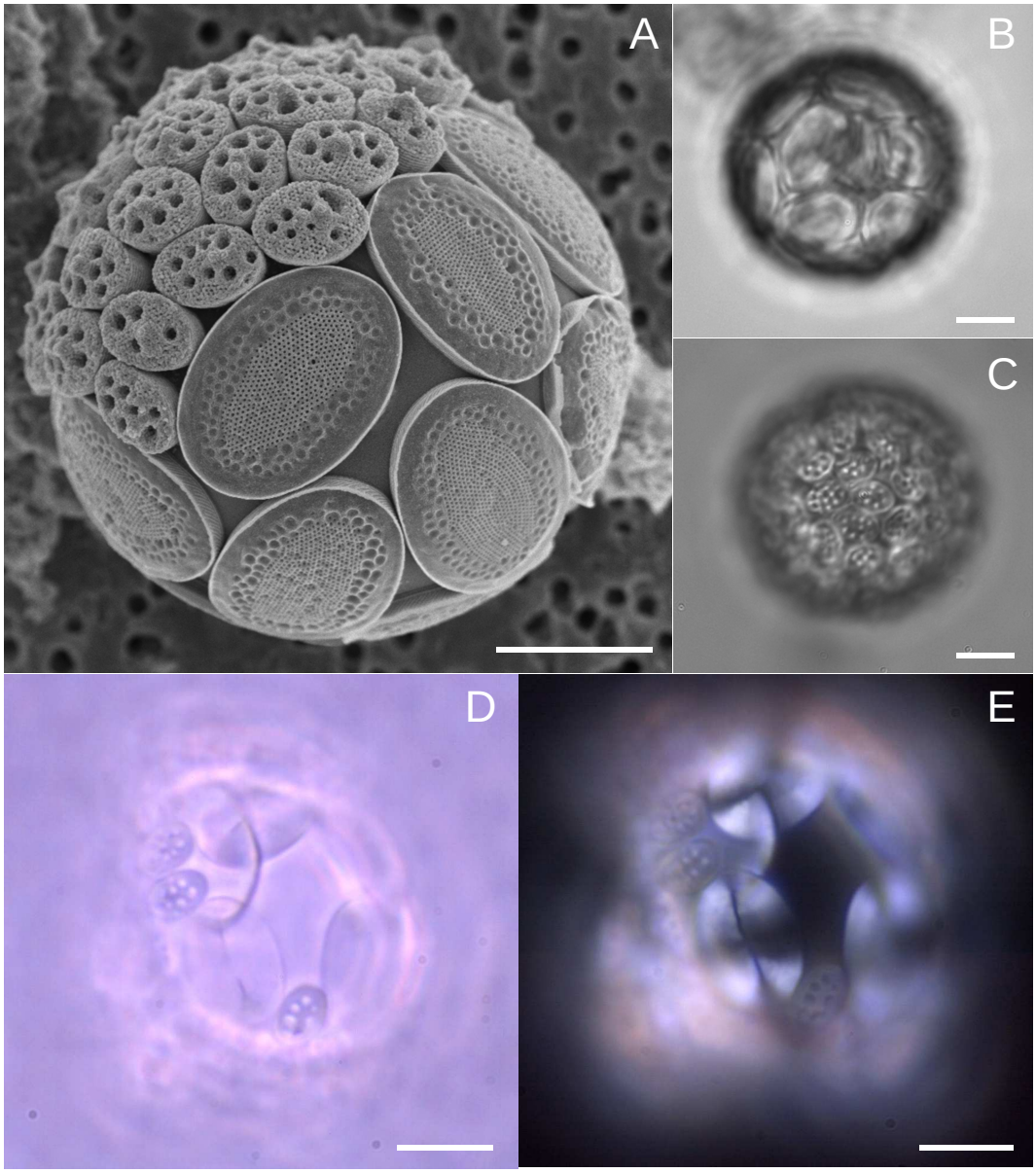


Figure 30. *P. japonic*a HET/HOL combination coccospheres. (A) SEM image of a combination coccosphere; Phase contrast images of (B) *P. japonica* HET, and (C) *P. japonica* HOL specimens; (B) Phase contrast and (D) cross polarization microscopy images of same combination coccosphere (scale bar = 5 µm).

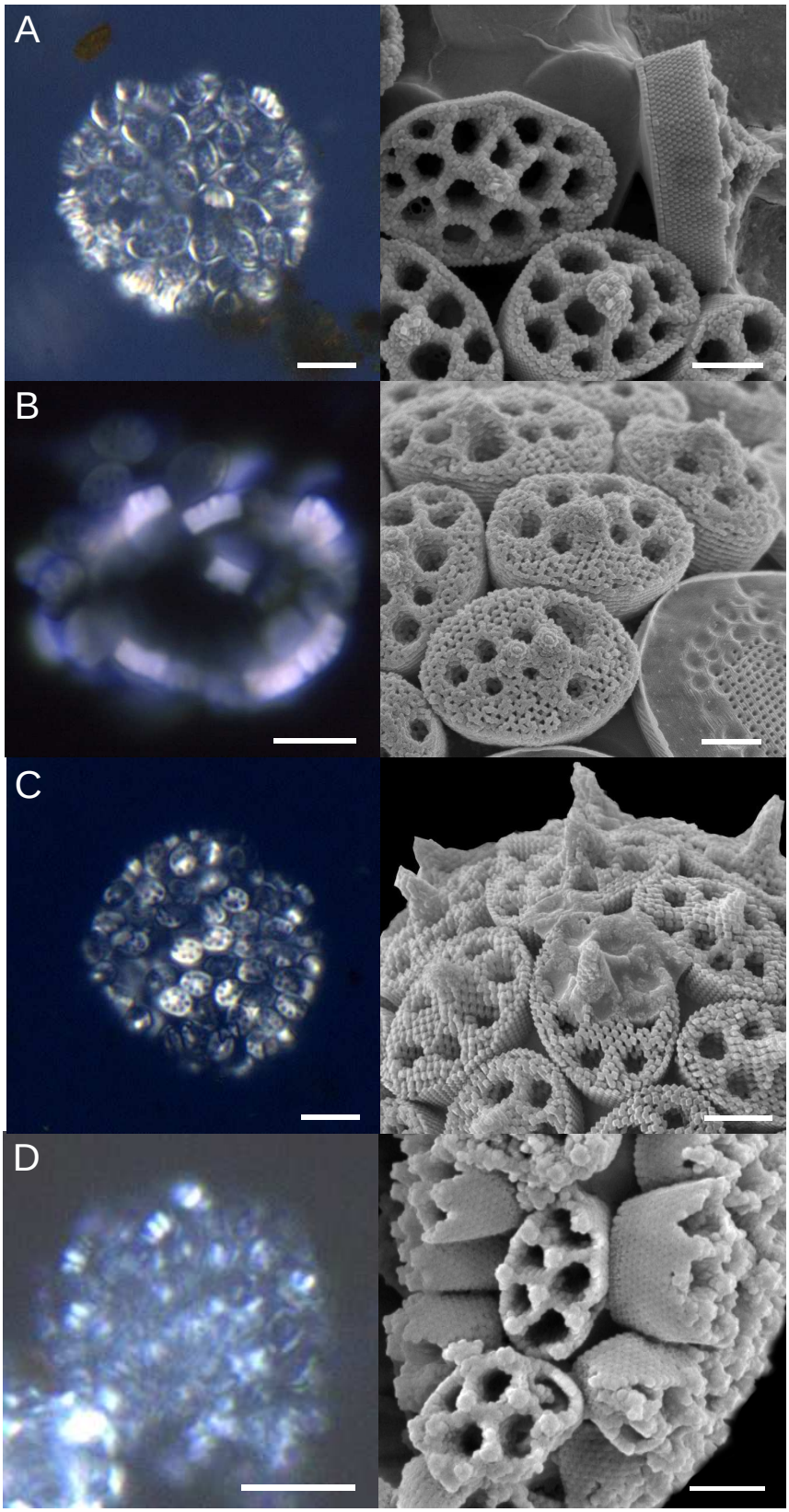


Figure 31. Cross-polarized microscopy images of species from the genus *Syracolithus* and SEM details of the respective holococcoliths. (A) *S. schilleri*; (B) *Pontosphaera japonica* HOL; (C) *Helicosphaera carteri* HOL (‘*S. confusus*’) and; (D) *Calcidiscus quadriperforatus* HOL (‘*S. quadriperforatus*’) (scale bars: left images = 5 µm; right images = 1µm).

4. Discussion

The rather regular coccolith arrangement of the *S. apsteinii – S. schilleri* combination coccosphere and the orientation of the two layers of coccoliths (Fig. 29) strongly suggest that this was a collapsed coccosphere and not some artefactual construction from a fecal pellet. This is further supported by the orientation of the equatorial lopadoliths at the edge of the coccosphere with the closed proximal surface pointing inward. The possibility that it was an accidental association as a result of two coccospheres colliding during filtration needs to be considered. However, the fact that the holococcoliths were interleaved with the heterococcoliths makes this unlikely. In addition, *Scyphosphaera* was very rare on the filter and no other specimen of *Syracolithus schilleri* was observed, thus the likelihood of an accidental association was extremely low. This *S. apsteinii – S. schilleri* association therefore almost certainly represents a transition between life cycle stages, analogous to those observed in several other coccolithophore species (e.g. (Cros et al. 2000)). In comparison, there is no doubt as to the authenticity of the *Pontosphaera japonica* -HET/HOL association since multiple coccospheres were found including one undamaged specimen (Fig. 30), which showed both holo- and heterococcoliths tidily arranged.

The two new holococcolith-heterococcolith associations presented herein are the first evidence for the occurrence of a dimorphic life cycle within the coccolithophore family Pontosphaeraceae. By analogy with previous observations in several coccolithophore families (Billard 1994, Houdan et al. 2004), the two stages found within each combination are related to a haplo-diploid life cycle, with holococcoliths being produced in the haploid phase and heterococcoliths in the diploid phase. The occurrence of a biphasic life cycle in the Pontospheraceae is not surprising, since this family belongs to the order Zygodiscales (Young & Bown 1997a) that includes *Helicosphaera* for which a heteromorphic haplo-diploid life cycle has already been recognized (Cros et al. 2000, Young et al. 2005). Nevertheless, the fact that similar *Syracolithus* morphotypes are assigned to highly divergent phylogenetic lineages is somewhat confusing. We further develop this issue in the next section.

4.1. The genus Syracolithus

The genus *Syracolithus* was created by Deflandre (Deflandre 1952) including the species *S. dalmaticus* and *S. catilliferus*. Additional morphospecies were subsequently placed in the genus by Loeblich & Tappan (Loeblich & Tappan 1963, 1966), Kleijne & Jordan (Kleijne & Jordan 1990) and Kleijne (1991). Kleijne (1991) emended the genus to include all

monomorphic holococcolithophores producing coccoliths formed of multiple layers of rhombic crystallites ('laminoliths'). High resolution electron microscopy and cross-polarised light observations showed that the species included in *Syracolithus* by Kleijne (1991) showed two very different types of structure (Young et al. 2003). The first structural group includes *S. catilliferus*, *S. dalmaticus*, *S. confusus* and *S. ponticuliferus*, in which the bulk of the coccolith consists of multiple layers of aligned rhombohedral crystallites with their crystallographic c-axes lying parallel to the plane of the coccolith (e.g. Fig 31C). There is also an outer wall, one crystallite thick, with the crystallites in hexagonal array and so probably with their c-axes sub-horizontal and pointing radially outward. In the light microscope the bulk of the coccolith typically behaves as a single calcite crystal, the outer-wall is usually not separately visible. From this group, *S. catilliferus* and *S. confusus* have been shown to be intergradational morphotypes both produced by the haploid life cycle phase of *Helicosphaera carteri* (Cros et al. 2000, Cros & Fortuño 2002, Geisen et al. 2002). The other species from this group (*S. dalmaticus* and *S. ponticuliferus*) have not yet been associated with a heterococcolithophore, however, due to close crystallographic and also morphological similarities, it is reasonable to predict that they may be produced by other *Helicosphaera* species (*H. hyalina*, *H. wallichii*, or *H. pavimentum*).

The second structural group of *Syracolithus* species, *S. quadriperforatus*, *S. bicorium*, *S. schilleri*, *S.* sp. A and *S.* sp. B, produce tube-like holococcoliths with the interior subdivided by internal septae (Fig 31A and D). These septae are formed of multiple layers of crystallites with their *c*-axes arranged vertically (i.e. perpendicular to the base-plane of the coccolith) and so appear dark in cross-polarised light. The outer wall, as in the other *Syracolithus* species, and almost all other holococcoliths, consists of crystallites arranged with their c-axes sub-horizontal and pointing radially outward, and so appears bright in cross-polarised light. The *Pontosphaera japonica* HOL found in this work is different from all *Syracolithus* morphotypes previously described, but shares some morphological characteristics with both *S. confusus* and *S. schilleri* (Fig. 31). The coccoliths resemble those of *S. confusus* in terms of shape and number of perforations, but are larger and have a wider central protrusion, often subdivided into two small protuberances, differing from the pyramidal, spine-like central knob found in *S. confusus*. In terms of size and crystallographic orientation, *P. japonica* holococcoliths match those of *S. schilleri*, thus showing affinity to the second group of *Syracolithus* species. However, the lack of an outer wall and the very characterisitic central protrusion make *P. japonica* HOL clearly distinguishable from both the above mentioned species.

In the second structural group, *S. quadriperforatus* was shown by Geisen et al. (Geisen et al. 2002) to form combination coccospheres with a large morphotype of *Calcidiscus leptoporus* (now renamed *Calcidiscus quadriperforatus*). It might be expected that *P. japonica* HOL and *S. schilleri* holococcoliths, which appear remarkably similar to those of *S. quadriperforatus* (Fig. 31), would belong to heterococcolithophore genera closely related to *Calcidiscus*. Our results are thus surprising, since both morphological (Perch-Nielsen 1985, Young et al. 1999) and molecular genetic (Saez et al. 2003, de Vargas et al. 2007, Medlin et al. 2008) data show that the closely related genera *Scyphosphaera* and *Pontosphaera* are only distantly related to *Calcidiscus* and its order, the Coccolithales, diverging since the early Mesozoic (ca. 200 Mya) according to palaeontological (Young et al. 1999) and molecular clock (de Vargas et al. 2007, Medlin et al. 2008) analyses . Hence the similarity between the holococcoliths of these distantly related genera appears to result from convergent evolution. A consequence of this is that it is presently difficult to predict whether *S. bicorium*, *S.* sp. A and *S.* sp. B are associated with other members of the Pontosphaeraceae or with Calcidiscaceae.

Our observations confirm the conclusion that holococcoliths are much poorer indicators of phylogenetic relationships than heterococcoliths (Young & Bown 1997b, Young et al. 2005) and suggest that they are under strong functional selection. For example, holococcoliths may be designed as protective shields to scatter UV radiation (Quintero-Torres et al. 2006) and thus allow holococcolithophores to thrive in shallower layers of the water column than heterococcolithophores. While diploid heterococcolith-bearing phases have become adapted to more specific and varied ecological niches, the haploid stages of various polyphyletic taxa may share common ecological strategies. On the other hand, homeomorphy could also be associated with the putative physical limitations in manipulating minute calcitic crystallites onto organic plate scales to form holococcoliths.

4.2. Geographic occurrence

The morphospecies *Scyphosphaera apsteinii* and *Syracolithus schilleri* are common but rarely abundant components of coccolithophore communities. Both life cycle stages have been reported in the Mediteranean Sea and the Atlantic and Pacific Oceans, notably from tropical and subtropical warm waters (Winter & Siesser 1994). In the Gulf of Naples long-term time-series samples, *S. apsteinii* recurs every year only during the cold months (November to February) (D. Sarno and A. Zingone, unpublished results). *Pontosphaera japonica* is much rarer, and has been reported in the Atlantic (Okada & McIntyre 1977,

Hallegraeff 1984, Mostajo 1984), Pacific (Reid 1980, Hallegraeff 1984), and sporadically in the Mediterranean Sea (Bartolini 1970, Young & Ziveri 2000). *P. japonica* HOL is apparently even rarer than its heterococcolithophore stage. In the literature, a single specimen morphologically similar to *P. japonica* HOL was reported from the Southern Tyrrhenian Sea as *Homozygosphaera schilleri* (Borsetti & Cati 1979).

4.3. Geological record

The geological record of holococcoliths is poor as they often disintegrate in the water column and the minute crystallites of which they are formed are readily overgrown or dissolved during diagenesis. Both holococcoliths discussed here should be relatively resistant since their structure of multiple layers of crystallites is relatively robust and since they are larger than average holococcoliths. Similar holococcoliths do indeed occur sparsely in the fossil record throughout the Neogene (Young 1998), and are most commonly recorded as *Holodiscolithus macroporus*. We used the Neptune database (Spencer-Cervato 1999) to examine the recorded occurrence of such holococcoliths and to see if they showed any correlation to occurrences of *Scyphosphaera* or *Pontosphaera* heterococcoliths, however the dataset proved too inconsistent to draw any useful conclusions. In the Palaeogene larger holococcoliths are more common and diverse assemblages have recently been described from Tanzania (Bown 2005b, Bown & Dunkley Jones 2006) including many forms with structure similar to the holococcoliths of *Pontosphaera* and *Scyphosphaera*. The stratigraphic range of such holococcoliths is not well-documented but the earliest records are from the Late Palaeocene, zone NP9, (Bown 2005a) and so co-incident with the first records of *Pontosphaera* heterococcoliths which are also from zone NP9 (Bybell & Self-Trail 1995, Bown 2005b) *Scyphosphaera* heterococoliths are first known form slightly later, in the Early Eocene (Siesser 1998, Bown 2005b). The similarity of the fossil range of the heterococcoliths and holococcoliths provides some support for the inference that the Pontosphaeraceae have consistently produced holococcoliths of this type and suggests that a more detailed study of their fossil record would be worthwhile.

4.4. Systematic taxonomy

Scyphosphaera apsteinii - Syracolithus schilleri

The species name *Scyphosphaera apsteinii* Lohmann 1902 has priority over *Syracolithus schilleri* (Kamptner 1927) Loeblich and Tappan 1963, as does the genus *Scyphosphaera* Lohmann 1902 over *Syracolithus* Deflandre 1952. *Scyphosphaera apsteinii* is therefore the appropriate name for both the heterococcolith-bearing and holococcolith-bearing phases of this species, which should henceforth be distinguished as *S. apsteinii* HET and *S. apsteinii* HOL following the convention of Young et al.(Young et al. 2003). In modern plankton this nomenclature can be applied with some confidence since the only other known extant *Scyphosphaera* species, *S. porosa* Kamptner 1967 is extremely rare (Young 2008). In the fossil record, however, numerous other *Scyphosphaera* species occur (Winter & Siesser 1994, Bown & Young 1998, de Vargas et al. 2007) and the known record of *S. apsteinii* type holococcoliths extends beyond the known fossil record of definite *S. apsteinii*. So in the fossil record it would be more appropriate to record this type of holococcolith as *Scyphosphaera* HOL than as *S. apsteinii* HOL.

***Pontosphaera japonica* HET/HOL**

Classically, *Pontosphaera japonica* HOL would have been described as a new species since it is distinctly different from any other morphotype described in the literature and it would have been assigned to the genus *Syracolithus*. The compelling evidence reported here that this holococcolithophore is a life cycle phase of the existing taxon *P. japonica* means a formal description is superfluous and the two entities within the life cycle should be distinguished as *P. japonica* HET or *P. japonica* HOL following the convention of (Young et al. 2003). An informal description of the HOL phase is given in Table 4.

5. Concluding remarks

The combination coccospheres documented here provide indirect but strong evidence that the genera Pontosphaeraceae exhibit haplo-diploid life cycles and this further supports the hypothesis that such a life cycle strategy is ubiquitous among calcihaptophytes. The recognition of coccolithophore life cycle associations is important for several reasons, particularly for developing a deeper understanding of the ecology of extant and extinct taxa, for further interpreting species evolution and relationships, and for predicting reactions of coccolithophore species *as a whole* (including both the haploid and diploid phases) to future

environmental changes. Presently ~30 life cycle associations have been discovered in this group (Young et al. 2003, Young et al. 2005). The low number of such observations compared to the total number of ~280 coccolithophore morphospecies (Young et al. 2003) results partly from the sporadic nature of sampling of open oceanic waters where most coccolithophore diversity is found. This contrasts with other groups of protists abundant in coastal waters for which sexuality has been much more widely observed, like dinoflagellates (Figueroa et al. 2006), and citations therein), or groups for which sexual stages are equally rare but which have been more extensively studied, like diatoms ((Assmy et al. 2008), and citations therein). The fact that very few combination coccospheres have been observed may also result from the relative rarity of sexual processes (syngamy and meiosis) in nature. Our discovery of several *P. japonica* combination cells in the same sample, with fewer cells of the same species bearing only heterococcoliths or holococcoliths, suggest that these phenomena may be very rapid and synchronous in a given population.. The ecological basis of sexuality and life cycles in coccolithophores remains poorly defined. A new approach that allows the combined genetic and morphological identification of single coccolithophore cells from natural samples ('COD-FISH') has recently been developed (Frada et al. 2006) and may prove to be extremely useful for linking life cycles phases and thus addressing specific questions related to the ecology and evolution of coccolithophore life cycles in the oceans.

Table 4. Summary of the principal characters of *Scyphosphaera apsteinii* HOL ('*Syracolithus schilleri*'), *Pontosphaera japonica* HOL, *Helicosphaera carteri* HOL ('*S. confusus*') and *Calcidiscus quadriperforatus* ('*Syracolithus quadriperforatus*').

Syracolithus schilleri	Holococcoliths with raised central area possessing 10-11 pores (rarely 15) disposed around a conical central protuberance; outer wall one crystallite thick showing hexagonal arrangement; coccoliths 3.3-3.7 µm long, 2.3-2.5 µm wide and 0.8-1.3 µm thick. In cross-polarized microscopy, the holococcoliths in plan view are characterised by a bright outermost wall forming a tube around a darker inner part, subdivided by faint internal septae.
Pontosphaera japonica HOL	Holococcoliths with a moderately flat top possessing a central ridge with one or two protuberances often surrounded by a smooth circular flange; 4-11 cavities of different sizes (0.3-0.8 µm diameter) disposed around the central boss; an outer wall is missing and the horizontally organized laminae are directly exposed to the exterior; coccoliths 3.5-4.0 µm long, 2.7-2.8 µm wide and 0.8-1.0 µm thick. In cross-polarized light the entire coccoliths appear very pale in plan view, but bright in side view.

Syracolithus confusus	Flat topped holococcoliths with a central ridge with one prominent and well defined tip surrounded by 7-10 pores; rim one crystallite wide showing hexagonal arrangement; coccoliths 2.3-2.9 µm long, 1.6-2.1 µm wide. In cross-polarized microscopy, the holococcoliths in plan view behave like a single crystal with dark pores disposed concentrically; the outer-wall is not separately visible.
Syracolithus quadriperforatus	Holococcoliths with irregular central area, possessing various short tips in the septae in crown-like configuration; 4-6 pores; outer wall one crystallite wide showing hexagonal arrangement; coccoliths 2-2.5 µm long, 2.0-2.5 µm wide and 0.8-1.1 µm thick. In cross-polarized microscopy, the holococcoliths in plan view are characterised by a bright outermost wall forming a tube around a darker inner part.

6. Acknowledgments

We would like to thank the Atlantic Meridional Transect program (AMT 16), namely Tony Bale as well as the officers and crew of the RRS Discovery. This work was supported by a US NSF grant DEB-0415351, an ATIP grant by the Centre National de la Recherche Scientifique (France). This work is part of MF's Ph.D. thesis, funded by the ''Fundação Para a Ciência e a Tecnologia'', Portugal, fellowship SFRH/BD/16851/2004, co-financed by the POCI 2010 and FSE. Research on coccolithophores from Tyrrhenian waters (IsaP) was supported by the project VECTOR (VulnErability of the Italian Coastlines and of the marine ecosystems to climate changes and their role in the oceanic caRbon cycle) funded by the Italian Ministry of University and Research. This work is part of the MarBEF EU FP6 Network of Excellence "Marine Biodiversity and Ecosystem Functioning" (contract no. GOCE-CT-2003-505446), and the pluridisciplinary project BOOM (Biodiversity of Open Oceans Microcalcifiers) funded by the French Agence National de la Recherche, grant ANR-05-BDIV-004.

7. References

Assmy P, Hernández-Becerril DU, Montresor M (2008) Morphologica variability and life cycle traits of the type species of the diatom genus *Chaetoceros*, *C. dichaeta*. Journal of Phycology 44:152-163

Bartolini C (1970) Coccoliths from sediments of the western mediterranean. Micropaleolontology 16:129-154

Billard C (1994) Life cycles. In: Green JC, Leadbeater BSC (eds) The Haptophyta Algae, Vol 51. Clarendon Press, Oxford, p 167-186

Borsetti AM, Cati F (1979) Il Nannoplancton calcareo vivente nel Tirreno centro-meridionale. Parte III. Giornale di Geologia 43:157-174

Bown P (2005a) Calcareous nannoplankton evolution: a tale of two oceans. Micropaleontology 51:299-308

Bown P, Young J (1998) Calcareous nannofossil biostratigraphy, Vol. Kluwer Academic Publishers, Cambridge

Bown PR (2005b) Palaeogene calcareous nannofossils from the Kilwa and Lindi areas of coastal Tanzania (Tanzania Drilling Project 2003-4). Journal of Nannoplankton Research 27:21-95

Bown PR, Dunkley Jones T (2006) New Paleogene calcareous nannofossil taxa from coastal Tanzania: Tanzania drilling project sites 11 to 14. Journal of Nannoplankton Research 28:17-34

Bybell LM, Self-Trail J (1995) Evolutionary, biostratigraphic and taxonomic study of calcareous nanofossils from a continuous Palaeocene-Eone buoundary section in New Jersey. U. S. Geological Survey professional paper 1554:1-36

Cortés MY, Bollman J (2002) A New Combination Coccosphere of the heterococcolith species *Coronosphaera mediterranea* and the holococcolith species *Calyptrolithophora hasleana*. European Journal of Phycology 37:145-146

Cros L, Fortuño J-M (2002) Atlas of Northwestern Mediterranean Coccolithophores. Scientia Marina 66:1-186

Cros L, Kleijne A, Zeltner A, Billard C, Young JR (2000) New examples of holococcolith-heterococcolith combination coccospheres and their implications for coccolithophorid biology. Marine Micropaleontology 39:1-34

de Vargas C, Aubry M-P, Probert I, Young J (2007) Origin and evolution of coccolithophores: from coastal hunters to oceanic farmers. In: Falkowski P, Knoll AH (eds) Evolution of Aquatic Photoautotrophs. Elsevier Academic Press, New York, p 251-285

Deflandre G (1952) Classe des Coccolithophoridés. (Coccolithophoridae. Lohmann, 1902). In: Masson (ed) Traite de Zoologie, p 439-470

Figueroa RI, Bravo I, Garces E, Ramilo I (2006) Nuclear features and effect of nutrients on *Gymnodinium catenatum* (Dinophyceae) sexual stages. Journal of Phycology 42:67-77

Frada M, Not F, Probert I, de Vargas C (2006) $CaCO_3$ optical detection with fluorescent in situ hybridization: a new method to identify and quantify calcifying microorganisms from the oceans. Journal of Phycology 42:1162-1169

Frada M, Probert I, Allen MJ, Wilson WH, De Vargas C (2008) The “Cheshire Cat” escape strategy of the coccolithophore *Emiliania huxleyi* in response to viral infection Proceedings of the National Academy of Sciences USA 105:15944–15949

Geisen M, Billard C, A.T.C. B, Cros L, Probert I, Young J (2002) Life cycle associations involving pairs of holococcolithophorids species: Intraspecific variation or cryptic speciation? European Journal of Phycology 37:531-550

Green JC, Course PA, Tarran GA (1996) The life-cycle of *Emiliania huxleyi*: A brief review and a study of relative ploidy levels analysed by flow cytometry. J Mar Syst 9:33-44

Hallegraeff GM (1984) Coccolithophorids (Calcareous Nanoplankton) from Australian waters. Botanica Marina 27:229-247

Houdan A, Billard C, Marie D, Not F, Saez A, Young G, Probert I (2004) Holococcolithophores-heterococcolithophores (Haptophyta) life cycles: flow cytometry analysis of relative ploidy levels. Systematics and Biodiversity 1:453-465

Kleijne A (1991) Holococcolithophorids from the Indian Ocean, Res Sea, Mediterranean Sea and North Atlantic Ocean. Marine Micropaleontology 17:1-76

Kleijne A, Jordan RW (1990) Proposed changes to the classification system of living coccolithophorids II. International Nannoplankton Association Newsletter 12:13

Loeblich AR, Tappan H (1963) Type fixation and validation of certain calcareous nannoplakton. Proceeding of the Biological Society of Washington 76:191-198

Loeblich AR, Tappan H (1966) Annotated index and bibliography of the calcareous nannoplankton. Phycologia 5:81-216

Medlin LK, Sáez AG, Young JR (2008) A molecular clock for coccolithophores and implications for selectivity of phytoplankton extinctions across the K/T boundary. Marine Micropaleontology 67:69-86

Moshkovitz S, Osmond K (1989) The optical properties and crystallography of Arkhangelskiellaceae and some other calcareous nannofossils in the Late Cretaceous. In: Crux J, Heck S (eds) Nannofossils and their applications. Ellis Horwood, Chichester, p 76-97

Mostajo EL (1984) Nanoplancton calcareo del Oceano Atlantico Sur. Revista Española de Micropaleontologia 17:261-280

Noel M-H, Kawachi M, Inouye I (2004) Induced dimorphic life cycle of a coccolithophorid, *Calyptrosphaera sphaeroidea* (Prymnesiophyceae, Haptophyta). Journal of Phycology 40:112-129

Okada H, McIntyre A (1977) Modern coccolithophores of the Pacific and North Atlantic Oceans. Micropaleontology 23:1-55

Perch-Nielsen K (1985) Cenozoic Calcareous Nannofossils. In: Bolli HM, Saunders, J.B. & Perch-Nielsen K (eds) Plankton Stratigraphy, Vol 1. Cambridge University Press, Cambridge, p 427-555

Poulton AJ, Adey TR, Balch WM, Holligan PM (2007) Relating coccolithophore calcification rates to phytoplankton community dynamics: Regional differences and implications for carbon export. Deep Sea Research Part II: Topical Studies in Oceanography 54:538-557

Quintero-Torres R, Aragón JL, Torres M, Estrada M, Cros L (2006) Strong far-field coherent scattering of ultraviolet radiation by holococcolithophores. Physical Review E 74

Reid FMH (1980) Coccolithophorids of the North Pacific central gyre, with notes on their vertical and seasonal distribution. Micropaleontology 26:151-176

Robinson C, Poulton AJ, Holligan PM, Baker AR, Forster G, Gist N, Jickells TD, Malin G, Upstill-Goddard R, Williams RG, Woodward EMS, Zubkov MV (2006) The Atlantic Meridional Transect (AMT) Programme: A contextual view 1995-2005. Deep Sea Research Part II: Topical Studies in Oceanography 53:1485-1515

Saez AG, Probert I, Geisen M, Quinn P, Young JR, Medlin LK (2003) Pseudo-cryptic speciation in coccolithophores. Proceedings of the National Academy of Sciences 100:7163-7168

Siesser WG (1998) Calcareous Nannofossil Genus *Scyphosphaera*: Structure, Taxonomy, Biostratigraphy, and Phylogeny. Micropaleontology 44:351-384

Spencer-Cervato C (1999) The Cenozoic Deep Sea Microfossil Record: Exploration of the DSDP/ODP Sample Set Using the Neptune Database. Palaeontologia Electronica 2:1-268

Thomsen HA, Østergaard JB, Hansen LE (1991) Heteromorphic life histories in artic coccolithophorids (Prymnesiophyceae). Journal of Phycology 27:634-642

Valero M, Richerd S, Perrot V (1992) Evolution of alternation of haploid and diploid phases in life cycles. Trends in Ecology and Evolution 7:25-29

Winter A, Siesser WG (1994) Atlas of living coccolithophores. In: Winter A, Siesser WG (eds) Coccolithophores. Cambridge University Press, Cambridge, p 107-159

Young G, Bown P (1997a) Cenozoic calcareous nannoplakton. Journal of Nannoplankton Research 19

Young G, Bown PR (1997b) Higher classification of calcareous nannofossils. Journal of Nannoplankton Research 19:15-20

Young J (1998) Neogene. In: Bown PR (ed) Calcareous Nannofossil Biostratigraphy. British Micropaleontological Society Publication Series. Chapman and Hall, p 225-265

Young J, Geisen M, Cros L, Kleijne A, Sprengel C, Probert I, Østergaard J (2003) A guide to extant coccolithophore taxonomy. Journal of Nannoplankton Research:125 pp.

Young JR (2008) *Scyphosphaera porosa* Kamptner, 1967 Rediscovered in the Plankton. Journal of Nannoplankton Research 30:35-38

Young JR, Davis SA, Bown PR, Mann S (1999) Coccolith ultrastructure and biomineralisation. Journal of Structural Biology 126:195-215

Young JR, Geisen M, Probert I (2005) A review of selected aspects of coccolithophore biology with implications for paleobiodiversity estimation. Micropaleontology 51:267-288

Young JR, Ziveri P (2000) Calculation of coccolith volume and its use in calibration of carbonate flux estimates. Deep-Sea Research Part Ii-Topical Studies in Oceanography 47:1679-1700

CHAPTER 5. Preliminary analyses of ploidy level in the Pavlovophyceae (Haptophyta)

Preliminary analyses of ploidy level in the Pavlovophyceae (Haptophyta)

Miguel Frada[1,2], Ian Probert[1], El-Mahdi Bendif[1], Colomban de Vargas[1]

[1]Station Biologique, Equipe EPPO-Evolution du Plancton et PaléOcéans, Centre National de la Recherche Scientifique et Université Pierre et Marie Curie (Unité Mixte de Recherche 7144), Station Biologique, 29682 Roscoff, France

[2]Departamento de Geologia, Faculdade de Ciências, Universidade de Lisboa, Edifício C6, Campo Grande, 1749-016 Lisboa, Portugal

Preliminary study

Abstract

Several species spanning the phylogeny of the haptophyte class Prymnesiophyceae have been shown to have haplo-diplontic life cycles, leading to the hypothesis that this life cycle type is ubiquitous in prymnesiophytes and possibly all haptophytes. However, presently there is no information about the life cycle type of the other haptophyte class, the Pavlovophyceae, that diverged from the Prymnesiophyceae early in the evolution of the haptophytes, several hundred million years ago. This knowledge would be valuable to unveil the origin and further understand the evolution and ecology of life cycles of haptophytes. In this preliminary study, flow cytometric analyses revealed a >twofold range of genome sizes in three Pavlovophyceae culture strains. Moreover, analysis of allozyme band patterns revealed that the strain with the largest genome size was diploid. Further analyses are required to determine whether haploid strains exist and hence to fully define the life cycle strategy of the Pavlovophyceae.

Key words

Haptophyta; Pavlovophyceae; Genome size; Ploidy; Life-Cycle

1. Intoduction

The Haptophyta are one of the most successful groups of phytoplanktonic eukaryotes in present oceans (Falkowski 2004). This division comprises more than 300 extant species that occur principally as solitary biflagellated unicells (from 2 to ~30µm in size). Other forms include colonies of motile cells, as well as non-motile cells that may be solitary or may form pseudo-filaments or mucous-bound aggregations (Jordan & Chamberlain 1997). These microalgae are widely distributed and abundant both in coastal and oceanic environments (Campbell et al. 1994, Not et al. 2005). Particular attention has been devoted to the ecology of certain haptophyte species due to the formation of toxic blooms (*Chrysochomulina, Phaeocystis*, *Prymnesium*, see (Houdan et al. 2004b) and references therein) and due to the significant role of some species (the coccolithophores) in marine calcification and hence carbon cycling (de Vargas et al. 2007, Iglesias-Rodriguez et al. 2008) or in the production of sulphur-rich climatically active molecules (see (Malin & Steinke 2004) and references therein).

According to molecular clock estimations, the Haptophyta is an ancient division that arose in the Precambrian more than 870 Ma (Yoon et al. 2004, de Vargas et al. 2007). In recent reconstructions of the eukaryotic tree of life, the haptophytes branch together with the cryptophytes (Hackett et al. 2007) at the base of the 'SAR' (Stramenoplies, Alveolates, Rhizaria) megaclade (Burki et al. 2007). Early in their evolutionary history the haptophytes diversified into two groups, the extant representatives of which are clearly distinct at the ultrastructural (Cavalier-Smith 1994, Green & Jordan 1994) and genetic (Edvardsen et al. 2000) levels: the Pavlovophyceae, a class containing <15 described species which exhibit relatively simple morphology (e.g. asymmetrical cell shape, anisokont flagella), and the much more intensively studied Prymnesiophyceae in which the majority of known extant haptophyte diversity is found, including the biogeochemically important coccolithophores (Edvardsen et al. 2000, de Vargas et al. 2007).

One of the most remarkable biological characteristics of the prymnesiophytes is their haplo-diplontic life cycle, with alternating haploid and diploid generations produced via the sexual processes of meiosis and syngamy, respectively (Billard 1994, Houdan et al. 2004a). Both phases are capable of producing autonomous populations by independent mitotic reproduction, and they can be morphologically very similar (isomorphy, e.g. *Prymnesium*) or clearly distinct (heteromorphy, widespread in the coccolithophores).

Current knowledge on sexual life cycles in the prymnesiophytes has originated mainly from comparison of clonal cultures that provide unambiguous evidence for the association of

two phenotypes with the exact same genotypic signature and allow determination of relative ploidy levels (e.g. Vaulot, Edvardsen, Houdan et al. 2004). Further indirect evidence has been obtained from the occasional observation of transitional coccolithophore morphotypes, possessing characteristic features (coccoliths) of both haploid and diploid phases (Frada et al. 2008). Maintenance of the physiological ability of alternating between, and growing asexually in, two ploidy levels expressing distinct phenotypes is likely linked to strategies for species to enlarge their ecological niches (Valero et al. 1992, Cros 2001, Thornber 2006).

The broad phylogenetic distribution of haplo-diplontic life cycles in prymnesiophytes suggests that this is probably the life cycle strategy common to all members of this class (Young et al. 2005, de Vargas et al. 2007). However, there is currently no information about life cyles of the Pavlovophyceae and the evolutionary origin of haplo-diponty in haptophytes is therefore unclear. Did this life cycle type evolve in the prymnesiophytes after divergence from the pavlovophytes? Alternatively, was it already present in the common ancestor of the two haptophyte classes? In this context, there are indications that at least some members of the sister group of the haptophytes, the cryptophytes, probably also possess a haplo-diplontic life cycle (Hill & Wetherbee 1986). This suggests that haplo-diponty may have been the ancestral state in the haptophyte-cryptophyte cenancestor that existed early in the history of the eukaryotes. In these terms, the strongest hypothesis would be that the Pavlovophyceae also undergo a haplo-diplontic life cycle, rather than either being asexual or having one dominant sexual phase as in the dinoflagellates (haplontic life cycle) or the diatoms (diplontic life cycle).

The present availability of approximately 40 culture strains of Pavlovophyceae (Roscoff Culture Collection: http://www.sb-roscoff.fr/Phyto/RCC/) constitutes an important pool of biological material for investigating this fundamental question. Here we present preliminary information from studies performed with three Pavlovophyceae strains, by light microscopy observations, flow cytometric estimation of DNA content, and ploidy level analysis through the study of allozyme band patterns. The results acquired during this study constitute the first steps in unveiling the life cycle strategy of this ancient microalgal group.

Technical note: allozymes and their utilization in this study

Allozymes are a variant of isozymes (multiple forms of an enzyme, expressed by different loci) that have the same catalytic specificity but slightly different gel migration rates. They are usually expressed co-dominantly, representing allelic alternatives of the same locus. Visualization of allozymatic band patterns thus allows recognition of the hetero- or

homozygous state of a particular marker and, by extension, can potentially be used to verify the ploidy level of an organism since only diploid organisms can have heterozygous patterns, whereas haploids will have only homozygous profiles for all markers (see (Manchenko 1994) and references therein).

This biochemical technique has classically been used by geneticists to study the population genetics of metazoans (Pasteur et al. 1987), fungi (Micales & Bonde 1995), higher plants (Gregg & Casey 2007), seaweeds (Sosa & Garcia-Reina 1992, Sosa & Lindstrom 1999)), and microalgae like diatoms (Gallagher 1982) and dinoflagellates (Baillie et al. 1998). It has also been used in the past to determine ploidy levels of fungi and other plant pathogens (Micales & Bonde 1995). This technique was employed in this study due to its availability in the laboratory of Evolution et Génétique des Populations Marines (EGPM) at the Station Biologique de Roscoff. A schematic explanation of the theory and interpretation of allozymatic bands is presented in the supplement material (SI 6).

2. Material and Methods

2.1. Culture growth conditions and microscopy

Non-axenic clonal algal strains (Table 5) were grown for 2 weeks in 3L of seawater (salinity 32) with K/2 (–Tris, -Si) medium supplements (Keller et al. 1987) and 0.1% (v/v) of soil extract at 20ºC and a light intensity provided by day-light fluorescent tubes of 150 µEinsteins.m^{-2}.s^{-1} with a photoperiod of 12:12 L:D.

Pavlovophyceae cultures were observed under an Olympus BX51 light microscope (Olympus, Tokyo, Japan) equipped for image acquisition with a RT-Slider Spot digital camera (Diagnostic Instruments, Sterling Heights, MI, USA).

Table 5. Haptophyte strains used in this study.

Strain code	Class	Species	Habitat	Collection site/ isolation date
RCC 1533 (=AC248)	Pavlovophyceae	*Pavlova* sp.	Marine Coastal	Mediterranean, St. Honora (France)/-
RCC 1537 (=AC44)	Pavlovophyceae	*Pavlova lutheri*	Marine Coastal	Baltic sea/-
RCC 1546 (=AC67)	Pavlovophyceae	*Diacronema vlkianum*	Marine Coastal, brackish water	English Channel/1986
RCC1347 (AC=101)	Prymnesiophyceae	*Isochrysis galbana*	Marine	Irish sea/-

2.2. Flow cytometry: DNA content estimation

1mL of exponentially growing culture was harvested and the nuclei were isolated, stained (SYBR-green), and processed for DNA content estimation by flow cytometry as described in (Marie et al. 2000). Analyses were performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, California, USA) equipped with an air-cooled laser providing 15mW at 488nm and with the standard filter setup. The *Isochrysis galbana* RCC1347 strain was used as an internal reference in order to allow direct comparison between the Pavlovophyceae strains.

2.3. Allozymes

The cultures for total protein extraction for allozymatic analyses were harvested by filtration (200 mmHg) using 2 or 3µm pore-size, 47mm diameter, polycarbonate filters (Osmonics Inc, Minnetonka, Minnesota USA) and rinsed thoroughly with sterile seawater in order to minimize bacterial biomass. The filters were then placed in ice-cold cryovials and covered with 1mL of cold extraction buffer (100 mM Tris-HCl, 5mM EDTA, 15 mM 2-β-NAD, 20% glycerol and 0.01% bromophenol blue) (Baillie et al. 1998). Total proteins were extracted using a French Press system (constant cell distribution system: www.constantsystem.com) at 1.70 psi. After extraction the samples were centrifuged (11000 rpm, 5 min., 4ºC) to remove debris and the supernatants aliquoted into new cryovials and frozen in liquid nitrogen. Long-term storage was conducted at -80ºC. Prior to analysis, the samples were thawed on ice. 45 µl of each extract was loaded (in triplicate) into a 20 cm long PAGE gel (electrode buffer: 0.025M Tris-HCl 0.19 M glycine, pH 8.3; stacking gel: 4% acylamide-bis-acylamide 0,5M Tris-HCl pH6.7; resolving gel: 7% acylamide-bis-acylamide 1.5M Tris-HCl pH 8.8). Electrophoreses were conducted in native conditions (no SDS or other denaturating agent was present) at 4ºC, 40mA for 16h.

After the electrophoretic migration the PAGE gels were incubated with 10 enzyme markers: glucose-phosphate isomerase (GPI); malate dehydrogenase (MDH); malic enzyme (ME); hexokinase (HK); isocitrate dehydrogenase (IDH); mannose-phosphate isomerase (MPI); esterase (EST); α-glucose-phosphate dehydrogenase (α-GPD); phosphate-glucose dismutase (PGM); superoxide dismutase (SOD). Allozymes were revealed through their activity to specific substrates, following classical methods described in Pasteur et al (Pasteur et al. 1987) (supplementray information 7). After revelation, an image of each gel was acquired with a Sony Cyber-Shot digital camera (Sony, Tokyo, Japan).

3. Results and Discussion

3.1. Microscope observations

The *Pavlova* sp. RCC 1533 culture (Fig. 32 A) contained actively swimming, round cells, each possessing two unequal flagella, one short and one long, as is characteristic in the Pavlovophyceae. Refringent droplets were often detected in the pole opposite the flagella (Fig. 32 A1). In the same culture, palmelloid colonies of many cells embedded in a common mucilage covering were also observed (Fig. 32 A2). These two cell types could constitute two ploidy levels or represent two morphological stages within the same ploidy level. Changes in morphology associated with changes in the ploidy level are common among prymnesiophytes, particularly among coccolithophores (Billard 1994), and sometimes both life cycle phases can be found together in the same culture, this being easier to detect when the two phases are heteromorphic. However, some species exhibit different morphologies within the same ploidy level. This is the case in the coccolithophore genus *Pleurochrysis*, in which the diploid phase is normally motile, but in old cultures or when plated on solid agar medium cells lose flagella and become flat, with multiplication leading to the formation of small colonies of juxtaposed cells (Gayral & Fresnel 1983). It is also the case for *Phaeocystis globosa*, where the diploid phase can be flagellated and free-living or form colonies embedded in a mucilage (Vaulot et al. 1994, Rousseau et al. 2007). Here, no attempt was made to isolate the two morphological types of this *Pavlova* sp. strain into pure culture. This approach would facilitate genetic analysis to confirm that both cell types originate from the same clone rather than resulting from cross-contamination, and would allow independent analysis of DNA content and comparison of ploidy levels.

The *Pavlova lutheri* RCC 1537 culture (Fig.32 B) only contained round, actively swimming cells, each possessing a green-brown parietal choroplast. In this strain the two flagella were both shorter than in the previous strain and were difficult to observe clearly (Fig. 32 B3). Highly refringent particles were present in the centre of the cell or close to the chloroplast.

The *Diacronema vlkianum* RCC 1546 culture (Fig.32 C) was also solely composed of actively swimming cells, and again the flagella were not easily observed. An orange stigma and spheres resembling lipid droplets were easily detected inside the cytoplasm, as previously described (Bendif 2006). As in the other strains, the presence of refringent structures was sometimes detected close to the green-brown parietal chloroplast (Fig. 32 C2).

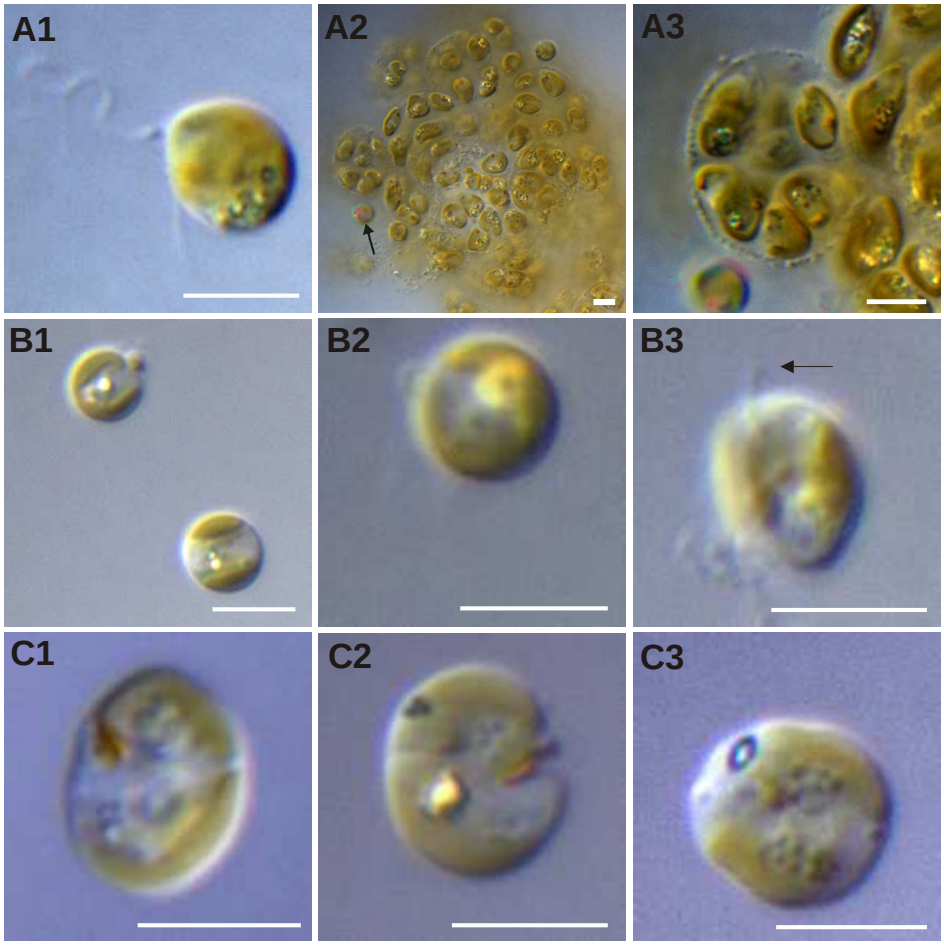


Figure 32. Light microscope images of the Pavlovophyceae strains used in this study.
(A1-A3) *Pavlova* sp. RCC 1533. In the culture there were solitary biflagellated cells (A1 and arrow in A2) and clumps of cells embedded in mucous (A2, A3).
(B1-B3) *Pavlova lutheri* RCC 1537. Solitary biflagellated cells, two short flagella (not always observable).
(C1-C3) *Diacronema vlkianum* RCC 1546. Biflagellated cells.

3.2. Flow cytometry: DNA content analyses

The DNA content of the three Pavlovophyceae strains was compared by flow cytometry. This analysis required the use of a reference strain to calibrate measurements of the test strains. *I. galbana* RCC 1347 was used as the reference strain because DNA content was within the required range and because precise and very reproductible measurements were obtained with isolated nuclei of this strain (Fig. 33 A). Nuclei were successfully isolated, stained, and analysed by flow cytometry for each of the Pavlovophyceae strains (Fig. 33 B to D).

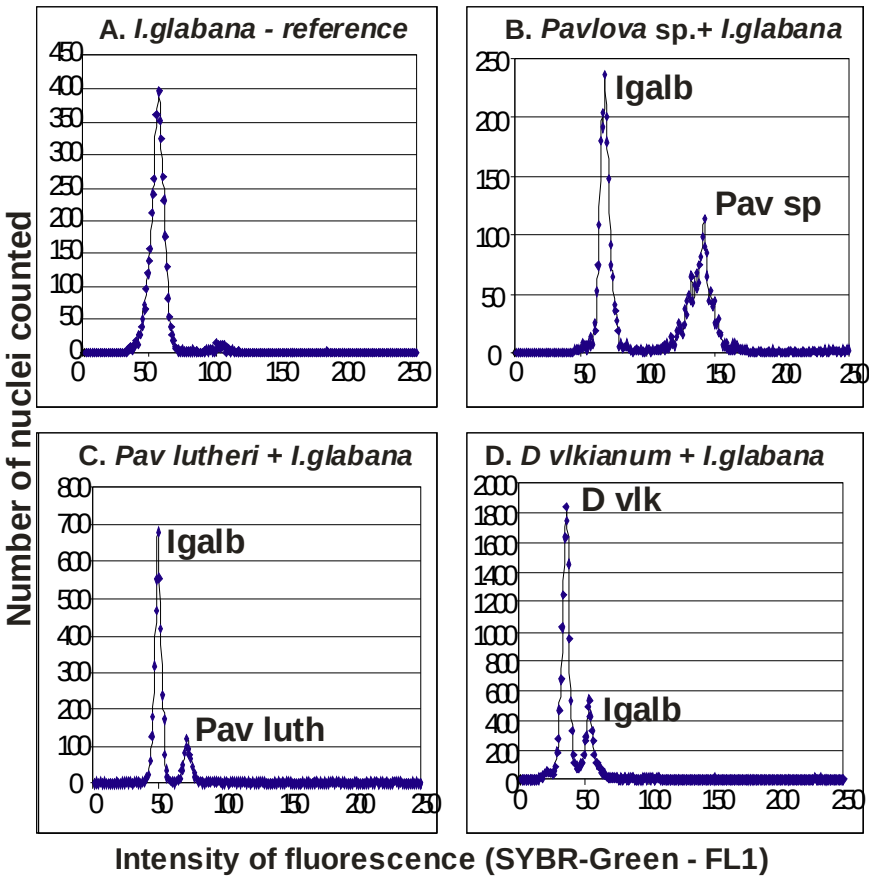


Table 2. Mean of the nuclei intensity

			Reference: *Igalbana*
A	*Pav* sp.	142	68
B	*Pav luth*	72	50
C	*D vlk*	36	54
D	*Iglabana*	G1: 57	

Figure 33. Relative DNA content of extracted nuclei determined by flow cytometry. **A**: *Isochrysis glabana* RCC 1347 (reference strain); **B** *Pavlova* sp. RCC 1533 and reference; **C** *P. lutheri* RCC 1537 and reference; **D** *D. vlkianum* RCC 1546 and reference.
Table 6. Mean values of the peak of fluorescence intensity of extracted nuclei.

RCC 1533 had the highest DNA content among the studied Pavlovophyceae, approximately twice that of *I. galbana*. RCC 1537 had a DNA content almost 50% higher than *I. glabana*, while RCC 1546 contained less DNA than *I. glabana* (Figure 34). There is, therefore, a >2-fold range of genome size variation between the Pavlovophyceae strains analysed. A possible explanation for this variation could be the presence of haploid and diploid strains. However, it is known from other organisms (including other microalgae) that genome size can vary widely among phylogenetically closely related species (Iglesias-Rodriguez et al. 2008), due to processes such as insertion of mobile elements, polyploidization, or loss of non-coding DNA (Cavalier-Smith 2005). According to our

phylogenetic data (E.M.Bendif and I.Probert, unpublished data), the three Pavlovophyceae strains used in this study are relatively distantly related and the genome size variation between them cannot therefore be conclusively assigned to ploidy level differences. For further studies, a more robust phylogeny based on more sequences would be useful to choose strains with closer affinities or even to identify life cycle phases of the same species that were isolated into clonal culture separately and originally identified as different taxa. This was the case for the discovery of the life cycle association of *Prymnesium parvum* - *P. patteliferum*, where phylogenetic analysis and then DNA content measurements led to the realization that these two 'species' were in fact life cycle stages of the same species (Larsen & Edvardsen 1998).

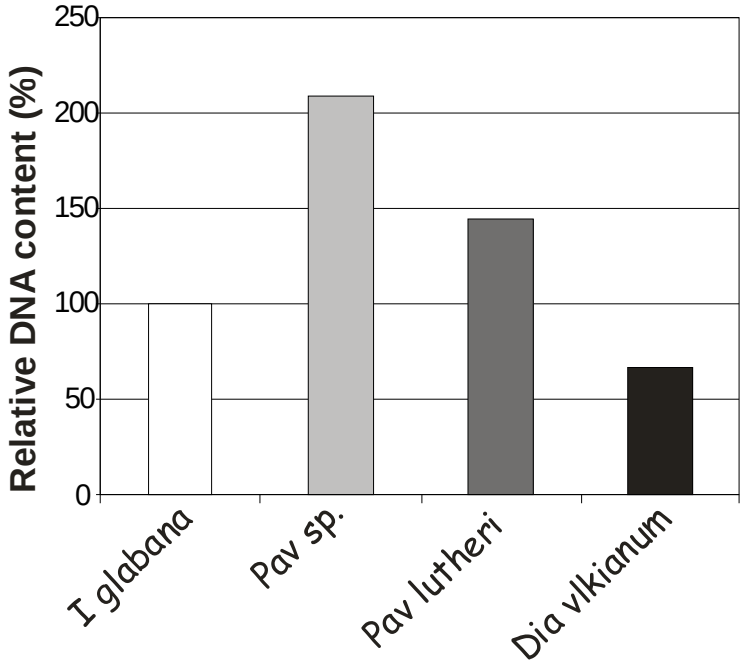


Figure 34. Pavlovophyceae DNA content relative to *Isochrysis glabana* (100%) determined by flow cytometry.

3.3. Allozyme analyses

The allozymatic profiles of the three species were analysed in order to check for the presence of homo- or heterozygotic markers which could be indicative of the ploidy level of the strains. The extract from *Pavlova* sp. strain RCC 1533 produced a close group of three bands for the glucose-phosphate isomerase (GPI) marker (Fig. 36 A). This means that the GPI

is a dimeric protein (composed of two sub-units) and that this strain is heterozygotic, possessing two alleles for the GPI (i.e. allele A: higher band; allele B: lower band; product of A and B: intermediate band). This strongly suggests that *Pavlova* sp. RCC 1533 is diploid, because in general hybrid bands are produced only by diploid organims (Sosa & Lindstrom 1999). For the malate-dehydrogenase (MDH) marker, three bands that were not equidistant were defined: the upper band was pale, followed closely by a stronger band and then a very strong lower band (Fig. 35 A). A possible interpretation is that the MDH could be a monomeric enzyme expressed by two loci (i.e. the bands represent isozymes). One isozyme could be heterozygotic (upper 2 bands) and the other homozygotic (lower band). Alternatively, the two sets of bands could represent MDH allozymes expressed by different parts of the cell, i.e. one set expressed by the nuclear genome and the other by an organellar genome. For the hexokinase (HK) and malate-phosphate isomerase (MPI) markers, only one band was detected, meaning that *Pavlova* sp. RCC 1533 is homozygotic for both markers. For the rest of the markers no signals were obtained from this strain (Fig. 35 A and Table 7).

For *Pavlova lutheri* RCC 1537 (Fig. 35 B), only one band was present for the GPI marker, indicating that this strain is homozygotic for this marker. In contrast, 4 bands (two groups of two bands) were obtained for MDH. The pattern is similar to that observed for *Pavlova* sp. RCC 1533, but here both isoforms would be heterozygotic. If this interpretation is correct, this strain would also be diploid, but further studies, namely the analysis of closely related strains (idealy strains of the same species) would be necessary to confirm if the pattern visualized during our study is common or artefactual. For all other markers no signals were obtained for this strain.

For *Diacronema vlkianum* RCC 1546 (Fig. 35 C), once again the GPI marker yielded one band, meaning that for this marker this strain is homozygotic. For the MDH, 2 bands were detected. Once again if we consider that this marker is expressed by two loci, this strain would be homozygotic for both (1 band per locus). For all other markers no signals were obtained for this strain.

All results of the allozymatic analyses are summarized in Table 7.

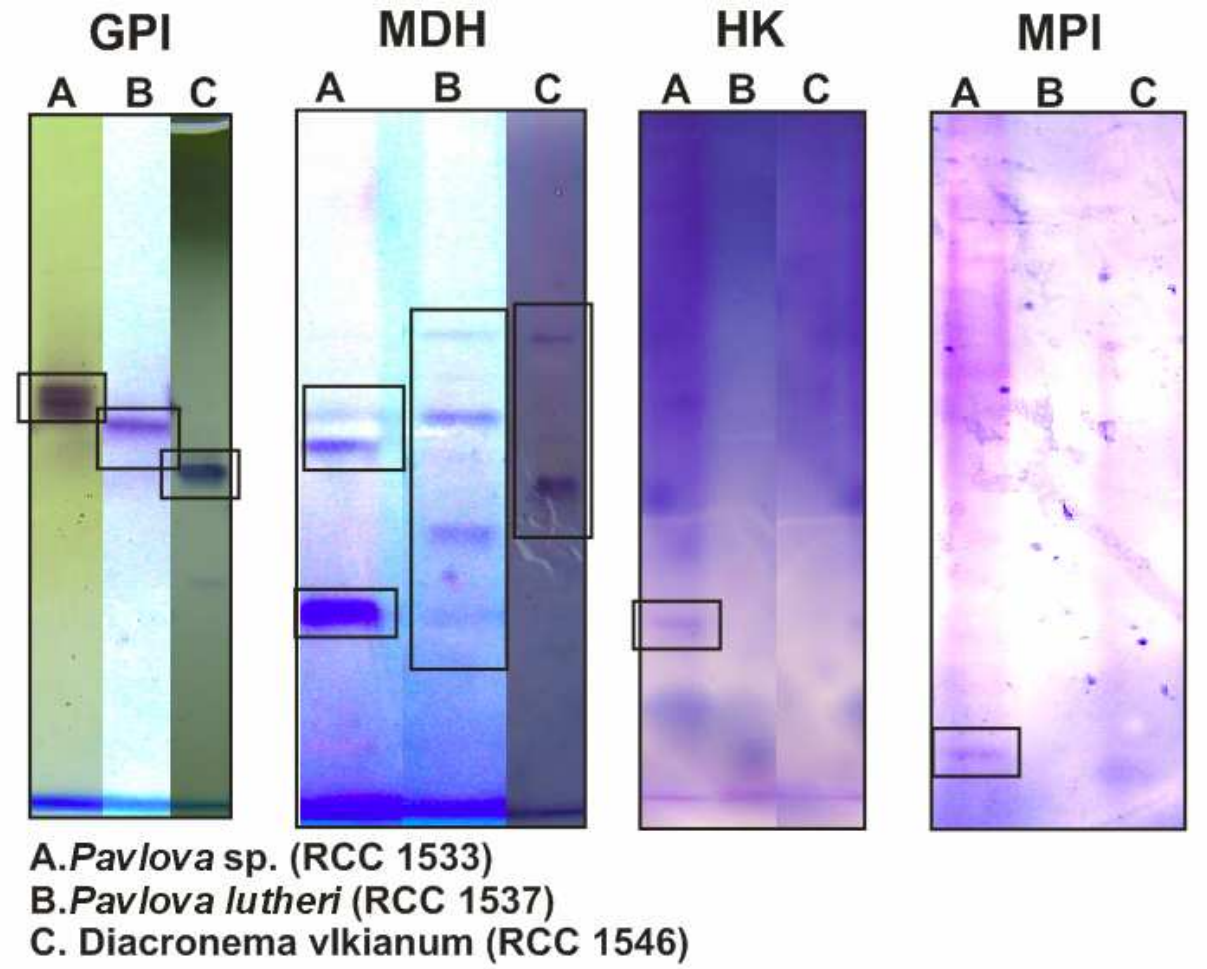


Figure 35. Allozyme gels (only markers for which positive results were obtained are shown).

Table 7. Summary of the allozyme results.

Allozymes	*Pavlova* sp. RCC 1533		*Pavlova lutheri* RCC 1537		*Diacronema vlkianum* RCC 1546	
GPI	3 bands	1 band	1 band	Homoz.	1 band	Homoz.
MDH	2 bands	Possibly 4 bands	Possibly 4 bands	X	2 bands	X
Malic enz.	No band	No band	No band	-	No band	-
HK	1 band	No band	No band	Maybe heteroz.	No band	-
IDH	No bands	No band	No band	-	No band	-
MPI	1 band	No band	No band	-	No band	-
EST	No band	No band	No band	-	No band	-
α-GPD	No band	1 band	1 band	-	No band	-
PGM	No band	No band	No band	-	No band	-
SOD	No band	No band	No band	-	No band	-

X see text

4. Conclusions and Prespectives

In summary, we found that there was a >twofold range of genome sizes within the Pavlovophyceae and we demonstrated that one strain (Pavlova sp. RCC 1533) was diploid. These constitute the first preliminary results towards the understanding of Pavlovophyceae life cycles.

According to this data, our main hypothesis that the Pavlovophyceae exhibit an haplo-diplontic life cycle has not yet been verified or rejected. Diploidy has been confirmed in at least one strain, which implies (but does not confirm) the existence of a sexual cycle in the Pavlovophyceae, and the existence of >twofold differences in DNA content (i.e. genome size) of the three strains also allows for the possible existence of two ploidy levels. However, comparison of DNA content of multiple strains of the same genotype will be required to potentially confirm this. At present, neither asexuality nor the existence of an exclusively diplontic cycle (i.e. two phases present with haploid phase cells serving only as gametes) in the Pavlovophyceae cannot be ruled out. Future studies should also take into account the possibility that other ploidy levels (tetraploid or polyploid) exist, which could complicate the analyses.

Allozyme analyses are potentially extremely useful for direct comparison of ploidy levels in protists, but have seldom been applied in this context and our experience shows that protocols need to be refined. The use of allozymes to identify the ploidy level of an organism when there is only one specimen (or clone) available, is in fact only possible when an organism is diploid and possesses recognizable heterozygotic markers, as was the case for the *Pavlova* sp. RCC 1533. If the clone is homozygotic for all markers studied there is a high chance, but no absolute proof, that the organism is haploid. However, if there is a high percentage of failure in the revelation of the allozymatic markers, further interpretation should be made with caution. During this study, no signals were obtained for the majority of allozymes tested. The reasons for this could be multiple:

- *Low efficiency of protein extraction:* the protocol used for extracting the allozymes was based on a protocol designed for dinoflagellates (Baillie et al. 1998), but it is also very similar to more general methods used in plants and macroalgae. In these terms there were no apparent reasons for it not to work for Pavlovophyceae, which have soft cells and do not have robust skeletons the presence of which could inhibit extraction. Moreover, we obtained well-

defined and concentrated protein bands in the case of the GPI marker, which seems to indicate that the extraction method was efficient.

- *Low intrinsic enzyme concentrations:* It is possible that under the conditions used to grow the cells some enzymes were less expressed than in natural conditions. This would affect the spectrum of allozymes present in these cells and would therefore affect the visualization of some (potentially all) markers. In addition, the cells were harvested for protein extraction at a given time of the cell cycle, when the specific expression of a given allozyme could be low. Alternative culture conditions or the collection of cells harvested at different times along the cell cycle could be tested in the future.

- *Degradation of allozymes during manipulation:* All extractions were performed on ice or at low temperature (≤4ºC). Moreover, the allozyme bands obtained were well-defined which indicates that there was no degradation during the various steps of manipulation.

- *Deficient electrophoretic conditions:* In general, in an electrophoretic gel under native conditions, migration and separation of the allozymes (or proteins in general) is based on their charge-to-mass ratio. The charge will be chemically modified under various conditions, including the pH of the electrophoresis buffers. In this study, only one migration and one gel buffer were used. This could have affected the direction of, or even impeded, migration of some of the allozymes. Alternative electrophoresis conditions should therefore be tested.

- *Revelation with non-specific substrates (or substrates for which Pavlovophyceae have low affinities):* The substrates and conditions used to reveal the allozymes were very general and have been shown to work for other microalgae. There are no obvious reasons for these not to work for analysis of extracts from Pavlovophyceae.

Proteins are the first result of the genetic expression of an organism. This expression is highly influenced by the conditions in which the organism is collected or grown. Moreover,

proteins are generally fragile molecules, more diffcult to handle than DNA. In these terms, alternative DNA-based methods that allow detection of different alleles within a clone should be tested in the future. One option would be the use of microsatellites, like those applied in a study of symbiotic dinoflagellates (Santos & Coffroth 2003). However, this method is laborious and requires the development of different genetic markers in order to produce consistent results. Alternatively, other DNA fingerprinting techniques like RAPD (random amplified polymorphic DNA), or even the analysis of single copy genes could be good candidate methods for the detection of different alleles. Other classical methods, like chromosome visualization and counting, could be also considered to determine the ploidy level of organisms. These methods have been used to determine ploidy of other haptophytes, for example *Hymenomonas lacuna* (Fresnel 1994). In fact one of our preliminary approaches consisted in attempting to visualize the chromosomes by epifluorescence microscopy from isolated nuclei (Fresnel 1994). However, we could not detect any chromosomes, probably due to their very small size.

Finally, another way to indirectly search for signs of life cycles (i.e. sexuality) would be to detect meiosis-related genes in the genome. This approach has been adopted to highlight the possible presence of sexuality in various protist taxa for which syngamy or meiosis had never been observed, e.g. the unicellular red algae, *Cyanidioschyzon merolae* (Malik et al. 2008). The impending availability of the first full genome sequence of a haptophyte (*Emiliania huxleyi*) and the present interest in sequencing the whole genome of Pavlovophyceae strains for fundamental (e.g. evolution) and applied (e.g. aquaculture) research (Nosenko et al. 2007) may provide a useful framework to design meiosis-related gene primers to search for sex related genes in the Pavlovophyceae.

5. References

Baillie BK, Monje V, V. S, M. S, Belda-Baillie CA (1998) Allozyme electrophoresis as a tool for distinguishing different zooxanthellae symbiotic with giant clams. Proceedings of the Royal Society B: Biological Sciences 265:1949-1956

Bendif EM (2006) Étude Cytomorphologique des Pavlovophyceae (Haptophyta). Université de Caen Basse-Normandie

Billard C (1994) Life cycles. In: Green JC, Leadbeater BSC (eds) The Haptophyta Algae, Vol 51. Clarendon Press, Oxford, p 167-186

Burki F, Shalchian-Tabrizi K, Minge M, Skjaveland A, Nikolaev SI, Jakobsen KS, Pawlowski J (2007) Phylogenomics Reshuffles the Eukaryotic Supergroups. PLoS ONE 2:e790

Campbell L, Shapiro LP, Haugen E (1994) Immunochemical Characterization of Eukaryotic Ultraplankton from the Atlantic and Pacific Oceans. Journal of Plankton Research 16:35-51

Cavalier-Smith T (1994) Origin and relationship of Haptophyta. In: Green JC, Leadbeater BSC (eds) The Haptophyta Algae, Vol Special volume No. 51. The Systematics Association, Oxford, p 413-435

Cavalier-Smith T (2005) Economy, Speed and Size Matter: Evolutionary Force Driving Genome Miniaturization and Expansion. Annuals of Botany (London) 95:147-175

Cros L (2001) Planktonic Coccolithophores of the NW Mediterranean. Universitat de Barcelona

de Vargas C, Aubry M-P, Probert I, Young J (2007) Origin and evolution of coccolithophores: from coastal hunters to oceanic farmers. In: Falkowski P, Knoll AH (eds) Evolution of Aquatic Photoautotrophs. Elsevier Academic Press, New York, p 251-285

Edvardsen B, Eikrem W, Green JC, Andersen RA, Moon-van der Staay SY, Medlin L, K. (2000) Phylogentic reconstruction of the Haptophyta inferred from the 18S ribosomal DNA sequences and available morphological data. Phycologia 39:19-35

Frada M, Probert I, Allen MJ, Wilson WH, De Vargas C (2008) The "Cheshire Cat" escape strategy of the coccolithophore *Emiliania huxleyi* in response to viral infection Proceedings of the National Academy of Sciences USA 105:15944–15949

Fresnel J (1994) A Heteromorphic Life Cycle in two Coastal Coccolithophorids, *Hymenomonas lacuna* and *Hymenomonas coronata* (Prymnesiophyceae). Canadian Journal of Botany 72:1455-1462

Gallagher JC (1982) Physiological Variation and Electrophoretic Banding Patterns of Genetically Different Seasonal Populations *Skeletonema Costatum* (Bacillariophyceae). Journal of Phycology 18:148-162

Gayral P, Fresnel J (1983) Description, sexualité et cycle de développement d'une nouvelle Coccolithophoracée (Prymnesiophyceae): *Pleurochrysis pseudoroscoffensis* sp. nov. . Protistologica 19:245-261

Green JC, Jordan RW (1994) Systematic History and Taxonomy. In: J.C.Green, Leadbeater BSC (eds) The Haptophyta Algae. Systematics Association Special Volume No. 51. Clarendon Press, Oxford, p 1-21

Gregg WW, Casey NW (2007) Modeling coccolithophores in the global oceans. Deep Sea Research Part II: Topical Studies in Oceanography 54:447-477

Hackett J, Yoon H, Li S, Reyes-Prieto A, Rümmele SE, Bhattacharya D (2007) Phylogenomic analysis supports the monophyly of cryptophytes and haptophytes and the association of rhizaria with chromalveolates. Molecular Biology and Evolution 24:1702-1713

Hill DRA, Wetherbee R (1986) *Proteomonas sulcata* gen. et sp. nov. (Cryptophyceae), a cryptomonad with two morphologically distinct and alternating forms. Phycologia 25:521-543

Houdan A, Billard C, Marie D, Not F, Saez A, Young G, Probert I (2004a) Holococcolithophores-heterococcolithophores (Haptophyta) life cycles: flow cytometry analysis of relative ploidy levels. Syst Biodivers 1:453-465

Houdan A, Bonnard A, Fresnel J, Fouchard S, Billard C, Probert I (2004b) Toxicity of coastal coccolithophores (Prymnesiophyceae, Haptophyta). J. Plankton Res. 26:875-883

Iglesias-Rodriguez MD, Halloran PR, Rickaby REM, Hall IR, Colmenero-Hidalgo E, Gittins JR, Green DRH, Tyrrell T, Gibbs SJ, von Dassow P, Rehm E, Armbrust EV, Boessenkool KP (2008) Phytoplankton Calcification in a High-CO2 World. Science 320:336-340

Jordan RW, Chamberlain AHL (1997) Biodiversity among haptophyte algae. Biodiversity and Conservation 6:131-152

Keller MD, Selvin RC, Claus W, Guillard RRL (1987) Media for the culture of oceanic phytoplankton. Journal of Phycology 23:633-638

Larsen A, Edvardsen B (1998) Relative Ploidy Levels in *Prymnesium parvum* and *P. patelliferum* (Haptophyta) analyzed by Flow Cytometry. Phycologia 37:412-424

Malik S-B, Pightling AW, Stefaniak LM, Schurko AM, Logsdon JM, Jr. (2008) An Expanded inventory of conserved meiotic genes provides evidence for sex in *Trichomonas vaginalis*. PLoS ONE 3:e2879

Malin G, Steinke M (2004) Dimethyl Sulfide Production: What is the Contribution of the Coccolithophores? In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Manchenko GP (1994) Handbook of Detection of Enzymes on Electrophoretic Gels, Vol. CRC Press, Boca Raton

Marie D, Simon N, Guillou L, Partensky F, Vaulot D (2000) Flow Cytometry Analysis of Marine Picoplankton. In: Diamond RA, DeMaggio S (eds) Living Color, Protocols in Flow Cytometry and Cell Sorting. Springer-Verlag, Berlin Heidelberg, p 421-454

Micales JA, Bonde MR (1995) Isozymes: Methods and Applications. In: Singh RP, Singh US (eds) Molecular Methods in Plant Pathology. CRC Press, Boca Raton

Nosenko T, Boese B, Battacharya D (2007) Pulsed-field gel electrophoresis analysis of genome size and structure in *Pavlova gyrans* and *Diacronema* sp. (Haptophyta). Journal of Phycology 43:763-767

Not F, Massana R, Latasa M, Marie D, Colson C, Eikrem W, Pedrós-Alió C, Vaulot D, Simon N (2005) Late summer community composition and abundance of photosynthetic picoeukaryotes in Norwegian and Barents seas. Journal Limnology and Oceanography 50:1677-1686

Pasteur N, Pasteur G, Bonhomme F, Catalan J, Britton-Davidian J (1987) Manuel Technique de Génétique par Électrophorèse des Protéines, Vol. Lavoisier, Paris

Rousseau V, Chrétiennot-Dinet M-J, Jacobsen A, Verity P, Whipple S (2007) The life cycle of Phaeocystis : state of knowledge and presumptive role in ecology. Biogeochemistry 83:29-47

Santos SR, Coffroth MA (2003) Molecular Genetic Evidence that Dinoflagellates Belonging to the Genus *Symbiodinium* Freudenthal are Haploid. Biological Bulletin 204:10-20

Sosa PA, Garcia-Reina G (1992) Genetic Variability and Differentiation of Sporophytes and Gametophytes in Populations of *Gelidium arbuscula* (Gelidiaceae: Rhodophyta) Determined by Isozyme Electrophoresis. Marine Biology 113:679-688

Sosa PA, Lindstrom SC (1999) Isozymes in macroalgae (seaweeds): genetic differentiation, genetic variability and applications in systematics. European Journal of Phycology 34:427 - 442

Thornber CS (2006) Functional Properties of the Isomorphic Biphasic Algal Life Cycle. Integrative and Comparative Biology 46:605-614

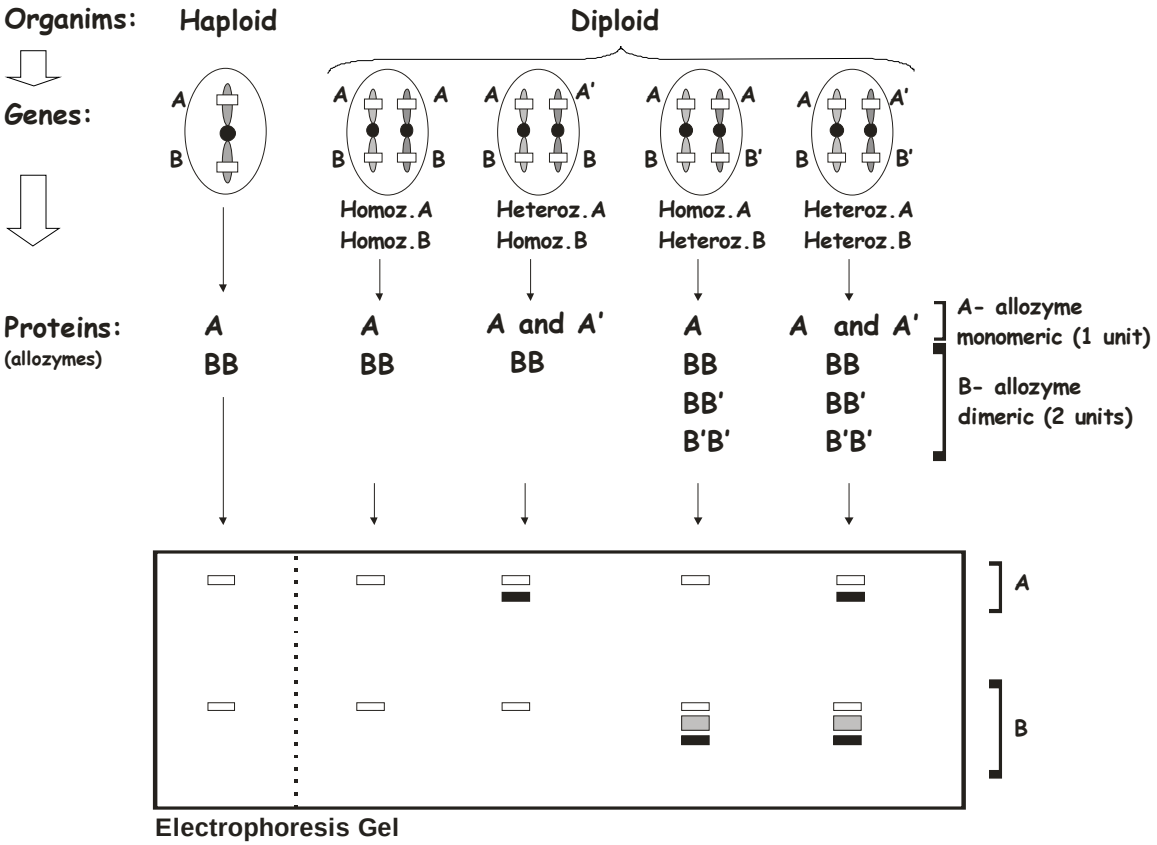
Valero M, Richerd S, Perrot V (1992) Evolution of alternation of haploid and diploid phases in life cycles. Trends in Ecology and Evolution 7:25-29

Vaulot D, Birrien J-L, Marie D, Casotti R, Veldhuis MJW, Kraay GW, Chretiennot-Dinet M-J (1994) Morphology, ploidy, pigment composition, and genome size of cultured strains of Phaeocystis (Prymnesiophyceae). Journal of Phycology 30:1022-1035

Yoon HS, Hackett JD, Ciniglia C, Pinto G, Bhattacharya D (2004) A molecular timeline for the origin of photosynthetic eukaryotes. Mol Biol Evol 21:809-818

Young JR, Geisen M, Probert I (2005) A review of selected aspects of coccolithophore biology with implications for paleobiodiversity estimation. Micropaleontology 51:267-288

Supplementary Information 6



Legend. Scheme of allozymes band pattern interpretation
Organisms can be haploid (one copy of the genome, and therefore one copy of each gene) or diploid (two copies of the genome and therefore two copies of each gene, i.e. one copy from the father and one from the mother). When an organism is diploid the father gene (called also father allele) can be genetically equal to the mother gene, and the new organism is homozygotic (A=A') for this gene. In opposition, when both parental genes are different, the new organims is heterozygotic (A≠A'). Then, the proteins (including the allozymes) expressed by these genes can be monomeric (consituted by one unit-A) or dimeric (consituted by two units- BB). When an organism is homozygotic for a monomeric protein, only one band is seen in a electrophoretic gel (A or A'). If it is heterozygotic, two bands are present (A and A'). When an organism is homozygotic for a dimeric protein it will also produce only two bands in an electrophoretic gel. However, if it is heterozygotic, three bands are present: one band representing the dimeric proteins produced by the same alelle (BB), another band representing the other alelle (B'B'), and in between these the product of both alelles (BB').
Haploid organisms are homozygotic for all genes. Diploid organisms can be homo- or heterozygotic for a given gene. The study of allozyme band patterns can be therefore used as a tool to recognize if an organism is haploid or diploid.*This simplified scheme can be even more complex, when proteins have more than 2 units (see (Pasteur et al. 1987, Manchenko 1994, Micales & Bonde 1995)).

Supplementary Information 7

Table A. Allozymes revelation protocols (Pasteur 1987)

Glucose-phosphate isomerase (GPI)	Coloration solution: Tris HCl 0.2M, pH8.0 – 10 ml $MgCl_2$ (0.5M) -1ml Fructose-6-phosphate- 10mg NAD 1% (in H_2O)- 1ml NADP 1% (in H_2O) – 0.5ml Just before use: Glucose-6-phosphate dehydrogenase (17 units)- 6µl PMS 1% - 0.5ml NBT 1% - 0.5ml MTT 1% - 0.5ml Mix with 1.5% agar in Tris A and cover the electrophoresis gel with the solution Incubate in the dark at 37ºC
Malate-dehydrogenase (MDH)	Coloration solution: Tris HCl 0.2M, pH8.0 – 10 ml $MgCl_2$ (0.5M) -1ml Malic acid (2M) pH7.0- 5ml NAD 1% (in H_2O)- 1ml Just before use: PMS 1% - 0.5ml NBT 1% - 1ml MTT 1% - 1ml Mix with 1.5% agar in Tris A and cover the electrophoresis gel with the solution Incubate in the dark at 37ºC (30-60 min)
Malic enzyme (ME)	Coloration solution: Tris HCl 0.2M, pH8.0 – 10 ml $MgCl_2$ (0.5M) -1ml Malic acid (2M) pH7.0- 1.5ml NADP 1% (in H_2O)- 0.1ml Just before use: PMS 1%- 0.2ml NBT 1%- 0.4ml MTT 1%- 0.4ml Mix with 1.5% agar in Tris A and cover the electrophoresis gel with the solution Incubate in the dark at 37ºC (30-60 min)
Hexokinase (HK)	Coloration solution: Tris HCl 0.2M, pH8.0 – 10 ml $MgCl_2$ (0.5M) -1ml α-(D)+Glucose - 500mg NAD 1% (in H_2O)- 1ml NADP 1% (in H_2O) - 0.5ml Just before use: Glucose-6-phosphate dehydrogenase (17 units)- 10µl PMS 1% - 0.5ml MTT 1% - 1ml Mix with 1.5% agar in Tris A and cover the electrophoresis gel with the solution Incubate in the dark at 37ºC
Isocitrate dehydrogenase (IDH)	Coloration solution: H_2O – 45ml Tris HCl 0.2M, pH8.0 – 25 ml $MgCl_2$ (0.5M) -1ml NADP 1% (in H_2O) – 1ml α-DL-isocitric acid – 170mg Just before use: PMS 1% - 1ml NBT 1% - 1ml MTT 1% - 0.5ml Incubate in the dark at 37ºC

Mannose phosphate isomerase (MPI)	Coloration solution: Tris HCl 0.2M, pH8.0 – 10 ml pyruvic acid -20mg D mannose-6-phosphate- 20mg NAD 1% (in H_2O)- 1ml NADP 1% (in H_2O) – 0.5ml Just before use: Glucose phosphate isomerase (10 units)- 10µl Glucose-6-phosphate dehydrogenase (17 units)- 6µl PMS 1% - 0.25ml MTT 1% - 1ml Mix with 1.5% agar in Tris A and cover the electrophoresis gel with the solution Incubate in the dark at 37ºC
Esterase EST	Coloration solution: Phosphate Na/Na2 0.1M, pH6.5 –100 ml [$Na_2H_2PO_4$, $2H_2O$- 9.98g; $Na_2H_2PO_4$, anhydric- 5.11g; H_2O- 100ml] α+βNaphtyl acetate 2% - 6ml [composition : α Naphtyl A- 0.5g ; β Naphtyl acetate A- 0.5g ; Acetone- 25ml ; H_2O- 25ml] Incubate at room temperature for 15 min Add Fast blue BB- 60mg
α-Glycerophosphate dehydrogenase (α-GPD)	Coloration solution: Tris HCl 0.2M, pH8.0 – 80 ml $MgCl_2$ (0.5M) -1ml α-DL-glycerophosphate - 200mg NAD 1% (in H_2O)- 2ml Just before use: NBT 1% - 1ml PMS 1% - 1ml MTT 1% - 0.5ml Incubate in the dark at 37ºC (60 min)
Phosphoglucomutase (PGM)	Coloration solution: Tris HCl 0.2M, pH8.0 – 10 ml $MgCl_2$ (0.5M) -1ml glucose-1-phosphate- 300mg NAD 1% (in H_2O)- 1ml Just before use: Glucose-6-phosphate dehydrogenase (17 units)- 6µl PMS 1% - 0.5ml NBT 1% - 0.5ml MTT 1% - 0.5ml Mix with 1.5% agar in Tris A and cover the electrophoresis gel with the solution Incubate in the dark at 37ºC (30-60 min)
Superoxyde dismutase (SOD)	Coloration solution: Tris HCl 0.2M, pH8.0 – 40 ml $MgCl_2$ (0.5M) -0.2ml NAD 1% (in H_2O)- 1ml Just before use: PMS 1% - 0.5ml NBT 1% - 1ml Incubate at room temperature with light, until the appearence of transparent bands in a blue background (1 to 2 hours)

PART IV. *GENERAL DISCUSSION AND PERSPECTIVES*

1. General discussion and perspectives

During this thesis diverse themes were explored around a core biological issue, **the haplo-diplontic life cycle of the Haptophyta**. The themes ranged from the physiology (*in vitro*) and ecology (*in situ*) of life phases of the ubiquitous coccolithophore ***Emiliania huxleyi***, including the development of a new method to identify and quantify calcifying and/or non-calcifying life cycle phases (**'COD-FISH'**), the discovery of two life cycles in the **Pontosphaeraceae**, a family branching within the core Calcihaptophycidae (see Fig. 6, Introduction), and finally the study of ploidy level of the **Pavlovophyceae**, a group that diverged early in the history of the Haptophyta (e.g. (Edvardsen et al. 2000)) and for which there was no previous information concerning the life cycle mode (Billard 1994). Given the time constraints, these themes are far from being inclusive, but together they provide an overview of life cycles in an important lineage of marine microalgae, emphasizing some topics without losing the notion of overarching issues. In this context, in this last section, the main results of the thesis will be highlighted and perspectives for future research in each topic will be proposed. Then, haptophyte life cycles will be discussed from a broader evolutionary point of view. Finally, further examples of potential applications of the COD-FISH method are presented.

1.1. The life cycle of *Emiliania huxleyi*

The major part of the research in this thesis was dedicated to study the life cycle of *E. huxleyi*. This species, like all other coccolithophores for which information exists, exhibits an heteromorphic haplo-diplontic life cycle. In *E. huxleyi* this life cycle is characterized by a calcified non-motile diploid phase and a non-calcified flagellated haploid phase bearing organic scales, but also a non-calcified non-motile diploid phase (Green et al. 1996) sometimes considered to be a culture artefact (Paasche 2001), but whose possible existence and role in the ecology of *E. huxleyi* should not be *a priori* neglected. Over the last three decades a plethora of studies have been dedicated to the calcified diploid phase that is clearly identifiable in water samples and easily amenable to laboratory culture, making it an outstanding marine biological model for eco-physiological and functional studies. By contrast, the non-calcifying phases have been almost completely overlooked both in field and laboratory research. Therefore, half of the life of this species, that is considered to be a key element of the marine biota, is unknown. In one of the rare studies dedicated to comparison of physiological responses of the diploid (calcified) and haploid phases of haptophytes, (Houdan

et al. 2005) reported that growth of cultures of the two phases was comparable (in terms of rate and yield) at non-saturating light intensities, but that photosynthesis of the diploid phase was optimal over a wide range of light intensities, whilst photosynthesis of haploid cells was inhibited at high light intensity. These results led to the suggestion that in the sea the two phases could exploit different ecological niches separated in space or time, as has been reported from field surveys of other coccolithophores (e.g. (Cros et al. 2000, Cros 2002)).

This hypothesis was further tested during this thesis, both through *in vitro* comparison of other ecophysiological characteristics of the two ploidy phases (**Chapter 1** and **Annex 4**) and through detection and quantification of the calcified and non-calcified phases in samples collected in various marine systems (**Chapter 3**, and **Chapter 2**).

In vitro (**Chapter 1**), we demonstrated that the haploid phase was not infected by viruses (*Emiliania huxleyi* viruses, *Eh*V) that infected and killed the calcified diploid phase, this potentially resulting from differences in the nature of the periplast between the two phases. We also observed the formation of a new haploid population after infection and complete collapse of a clonal culture of diploid calcified cells. These results suggested that biotic interactions with viruses could trigger life cycle changes, or at least promote the ecological succession between life cycle phases in nature. We coined this process 'Cheshire cat' dynamics, after the Cheshire cat in *Alice's Adventures in Wonderland* (Carroll 1865) that made its body invisible to escape the sentence "off with his head'' pronounced by the Queen of Hearts. In this context an ecological role of the resistant haploid phase could be to escape highly infectious *Eh*Vs that are responsible for the termination of natural blooms (Jacquet et al. 2002, Wilson et al. 2002). The phase change process would release, to some extent, *E. huxleyi* from pathogen pressure and from the need to evolve resistance in the diploid phase in order to maintain fitness in a long-term arms race with the viruses, as the classical Red Queen model predicts (Van Valen 1973, Otto & Lenormand 2002). Instead, refuge in the haploid phase would allow *E. huxleyi* to evolve in other directions, notably maintaining its eco-physiological competitivity in the diploid phase. Finally, this process could be responsible for active selective maintenance of the biphasic sexual life cycle in *E. huxleyi* and the evolution of distinct morphologies in each phase.

These results and the theory proposed to interpret them raise numerous new questions and open diverse perspectives for further research. Firstly, it would be interesting to see whether Cheshire cat dynamics can be detected in other host-virus systems, notably those in which the host exhibits a haplo-diplontic life cycle, including other coccolithophores and haptophytes, but also other organisms like many multicellular red and brown algae

(seaweeds). Certain of these have clear potential to be used as models for pursuing this issue, like for example the brown macroalga *Ectocarpus siliculosus* which has an isomorphic haplo-diplontic life cycle (Muller 1977, Bell 1997, Coelho et al. 2007) and for which specific viruses have been described and isolated (Müller et al. 1990, Delaroque et al. 2001). In a broader context, this theory could also be applicable to any host-pathogen or predator-prey interaction and we hope that it will stimulate debate in these fields.

There is also considerable scope for further investigation on specific themes related to host-pathogen interactions, for example analysis of biochemical properties of diploid and haploid cells to detect putative recognition molecules exploited by EhVs (see (Lenski 1988) and references therein), and study of the mechanism(s) that promote meiotic transition from the diploid to the haploid phase in response to viral activity (for instance the potential role of oxidative stress and/or inter-cellular signalling mechanisms). This would first require a careful examination of infection dynamics in order to pinpoint the timing of meiosis of diploid calcified *E. huxleyi* cells. Then further experimentation would be required to elucidate the possible cues triggering this response. For example, an experimental approach that could be pursued would be to study of the effect of varying the concentration of reactive oxygen species (ROS) that are recognized as triggers of sex in other algae (Nedelcu et al. 2005) and are known to be produced by *E. huxleyi* during EhV infections (Evans et al. 2006).

In this thesis, the next step (related to seeking how Cheshire cat dynamics might operate in natural populations) was to explore the prevalence of both life cycle stages of *E. huxleyi* in various marine ecosystems including blooms, where EhVs are naturally present and play a key role in the regulation and termination of host population development (Bratbak et al. 1993, Castberg et al. 2001, Jacquet et al. 2002, Wilson et al. 2002, Martinez et al. 2007). For this field approach we developed the COD-FISH method that links the genetic signature and the morphology of the targeted organism(s), allowing distinction of calcified from non-calcifed forms of *E. huxleyi* (this method has various potential applications to study other coccolithophores and calcareous organisms in natural samples, as exposed in **Chapter 2** and regularly highlighted during this final discussion).

In field surveys (**Chapter 3**), we observed that calcified and non-calcified phases of *E. huxleyi* co-existed in the same water masses and followed similar population dynamics over time. However, the diploid calcifed phase was generally dominant, whereas the non-calcifed phase was present at lower background concentrations (5-40% of total *E. huxleyi* cells). We did not observe seasonality in the distribution of the two forms. Moreover, we developed a

method to detect haploid flagellated cells among the non-calcified *E. huxleyi* population and applied this to samples from the mesocosm experiment. Non-calcified cells were detected both by light and electronic microscopy and by quantitative epifluorescence microscopy of DAPI stained cells. Based on this analysis, we suggested that diploid and haploid cells of *E. huxleyi* may always be present together in wild populations and that this coexistence could be the basis of the ecological success of this species. This hypothesis requires validation by monitoring of more locations where *E. huxleyi* is present throughout the year, paying particular attention to the post-bloom period after the demise of diploid cells mediated by *Eh*Vs. In addition to the use of COD-FISH, other molecular genetic techniques, like real-time quantitative-PCR (rtQ-PCR) targeting genes specifically expressed in the haploid phase or genes involved in meiosis or cell-cell recognition (as discussed in Chapter 3), should be applied in order to define precisely the prevalence within the non-calcified background population of haploid cells and highlight the rate and timing of sexual turnover in nature.. Real-time Q-PCR is a very sensitive method that has been used in recent years to detect and quantify various phytoplankton species (particularly toxic species) in environmental sampes (Bowers et al. 2000, Coyne et al. 2004, Zhu et al. 2005, Parke et al. 2007, Amato 2008). With this technique, a target gene is amplified with specific primers and product formation is monitored after each PCR cycle (in real-time) by measuring a fluorescence signal. If the efficiency of the reaction remains constant, the increase in fluorescence observed during the reaction will be proportional to the starting quantity of the target gene, which, after calibration for a given species, correlates well with the number of individual cells of that species present in the sample (Penna & Galluzzi 2007). This approach would lead to a better understanding of the population dynamics and the ecological role of both life cycle phases. Moreover, it would provide a better indication of the complete biogeochemical impact of *E. huxleyi*, including its hidden non-calcified life cycle phases.

In parallel to the acquisition of these field results, a preliminary study was performed in the frame of the Masters thesis of Daniella Mella-Flores (**Annex 4**), in which diploid (calcified) and haploid cells were mixed in the same culture flasks at different relative proportions (25%-diploid/75%-haploid; 50%-diploid/50%-haploid 75%-diploid/25%-haploid) and growth of each phase monitored over time. In these experiments diploid cells tended to become numerically dominant over haploid cells even when initially inoculated at lower concentrations. Furthermore, we tested for potential allelopathic effects of one phase over the other by switching cells to a medium in which the other life cycle phase had been growing

exponentially. In this experiment no differences were observed in the growth of culture populations following this treatment. However, measurement of nitrate and phosphate consumption rates provided evidence that diploid cells took up nitrate more rapidly, which revealed a potential competitive advantage of diploid cells in mixed cultures. These new *in vitro* experiments were only preliminary, but results were generally consistent with (and provide some insights into) the observation that calcified diploid phase appears to dominate haploid cells in the natural environment.

Further investigation is needed both in the field and the laboratory to consolidate the conclusions presented here concerning the ecological role and physiological differentiation between the diploid and haploid phases of *E. huxleyi*. This species is capable of haplo-diplonty (i.e. both phases can develop asexually), but our results indicate a clear competitive (and resulting numerical) dominance of the diploid phase. One implication of this is a strong spatial proximity between the two phases, as we observed. This apparent bias towards one phase represents a deviation from the classical concept of a haplo-diplontic life cycle in haptophytes where both phases are considered capable of forming independent and equally important populations (in terms of numbers and/or time).

One area of research that could prove directly (and will most probably prove) relevant to the investigation of the life cycle of *E. huxleyi* is the field of genomics. This new approach, highly complementary to the research presented here, has the potential to provide new insights into the life cycle of *E. huxleyi*. A comparative study of the genomics of haptophyte life cycles is presently being undertaken by P. van Dassow at the Station Biologique de Roscoff in collaboration with C. de Vargas and I. Probert (CNRS, Station Biologique de Roscoff) and H. Ogata (CNRS, Marseille). The core objective of this study is to compare the transciptomes (Sanger and 454 sequencing of normalized clone libraries) of diploid and haploid cells of several species, including *E. huxleyi*, in order to reveal differences in gene expression between ploidy stages. This will lead on to functional analysis, aiming to reveal which genes are activated under given environmental conditions to further characterize physiological differentiation between the two ploidy phases. Ultimately, this work will reveal many aspects of the genetic basis of the life cycle of *E. huxleyi* (and other coccolithophores) and the data produced will promote a number of new applications, including for example the design of genetic probes to target and/or quantify (FISH, Q-PCR, etc.) each life cycle phase in nature.

Longer term, a common aim of the *E. huxleyi* research community is to develop methods to genetically transform this species, which would open another wide array of perspectives for future research. For instance, there is currently great interest in, but little understanding of, the genetic mechanisms that regulate the process of calcification (Brownlee & Taylor 2004). These might be elucidated through utilization of mutant strains lacking the ability to calcify.

In terms of research on life cycles, an essential step will be mastering the complete manipulation of the sexual cycle of *E. huxleyi*. because it permit a full manipulation of *E. huxleyi* strains, allow the engineering of specific crosses to unveil mutant phenotypes that would be of value for both fundamental (functional studies, evolutionary biology) and applied (over-expression of a given metabolite) purposes. One study reported the induction of meiosis in this species after switching diploid cells from liquid to solid culture medium (Laguna et al. 2001). However, these results have proved impossible to reproduce, and moreover, this study did not provide reliable cytological evidence for the presence of haploid cells, and consequently this is generally considered by the scientific community interested in this theme as a result that should be considered with caution (Houdan et al. 2004). To our knowledge, crossing experiments between different haploid *E. huxleyi* culture strains have never been carried out. Future studies on the processes of syngamy and meiosis in this species should make reference to those on the model chlorophyte genus *Chlamydomonas*, in which cell recognition factors and mating systems have been extensively investigated (Mitchell 2000, Harris 2001).

1.2. Calcihaptophyte life cycles

Within the core group of the calcihaptophytes, we unveiled the first two HET-HOL life cycle combinations in the family Pontosphaeraceae, i.e. the life cycles of *Scyphosphaera apsteinii* and of *Pontosphaera japonica* (**Chapter 4**). Following the predictions of Billard (Billard 1994) and Houdan et al. (Houdan et al. 2004), these are indicative of the existence of a haplo-diplontic life cycle, with holococcoliths being produced in the haploid phase and heterococcoliths in the diploid phase. These observations provide further support for the hypothesis that the haplo-diplontic life cycle trait is common to all calcihaptophytes, since practically all families have a few representative species for which both life cycle phases have already been recognized ((Young et al. 2005) and references therein). There are, however, some lineages for which information on life cycle strategy is still lacking (see Fig. 6, Introduction). Of these, the Calciosolenaceae is thought to be very closely related to the

Syracosphaeraceae and the Rhabdosphaeraceae, both of which possess recognized haplo-diplontic (HET-HOL) life cycles. It is therefore reasonable to predict that species belonging to the Calciosolenaceae also possess a haplo-diplontic life cycle with HET and HOL phases. Another intriging group are the Calyptrosphaeraceae, composed of several holococcolithophore species which form a distinct phylogenetic group and for which only one HET phase of one species has been observed after culture manipulation (Noel et al. 2004). Perhaps the largest remaining uncertainty concerning calcihaptophyte life cycles concerns the Umbellosphaeraceae, characterized by species that colonize highly oligotrophic mid-oceanic areas, and which do not currently have a defined position in the Calcihaptophycidae phylogeny. It would be interesting to unveil the life cycle strategy exhibited by members of this family to adapt to a permanently nutrient-poor environment.

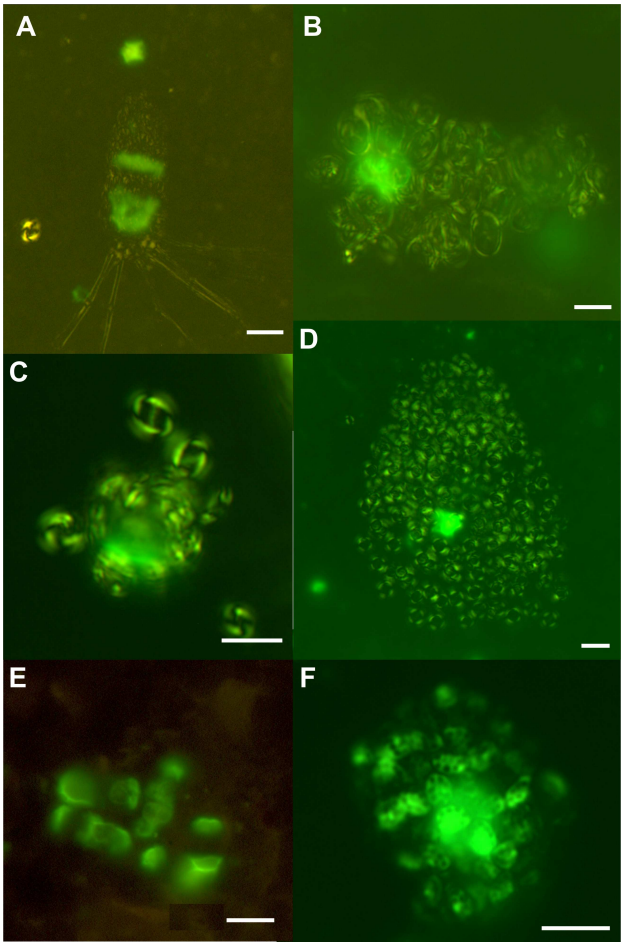


Figure 36. Example of the different coccolithophores analysed by COD-FISH using the general haptophyte probe Prym-02 (Simon et al. 2000). The coccosphere is visible by cross-polarization microscopy and in the centre the cell labelled with Prym-02 is highlighted in green (scale = 5 µm). A. *Michaelsarsia* sp., collected in the west Pacific; B. *Syracosphaera pulchra*, collected in Villefranche sur Mer (France); C. *Gephyrocapsa oceanica*, collected in the west Pacific; D. *Umbilicosphaera sibogae*, collected in the west Pacific; E. Benthic aggregation (or colony) of at least nine non-calcified haptophyte cells, collected in the sand during low tide at Roscoff (France); F. *Helicosphaera carteri* HOL haploid phase (ex. *Syracolithus confusus*), collected in Villefranche sur Mer (France).

Further monitoring of water samples, exploring more marine environments should therefore be pursued. Since observations of combination coccospheres are rare, the COD-FISH method (Fig. 36) with specific oligonucleotidic probes should be exploited to reveal HET-HOL associations. Barring culture observations, COD-FISH will be the only viable method for unveiling life cycle associations in species where one of the life cycle counterparts proves to be non-calcified, like in *E. huxleyi*, remaining therefore invisible to classical microscopy (light microscopy and scanning electronic microscopy) methods ((de Vargas & Probert 2004) and see Figure 17 in Chapter 2).

In my opinion, a research strategy that will highlight and clarify a wide range of issues concerning the significance and adaptive advantages of the biphasic haplo-diplontic, heteromorphic (and heterophysiological) life cycle in the calcihaptophytes, will be ecological studies performed through long term monitoring of given sampling stations, where an abundant community of species is commonly present and where both HET and HOL phases are commonly observed (e.g. Mediterranean sea). Such an approach has provided invaluable information in classical studies of the life cycles of diatoms (e.g.(Mann 1988, Assmy et al. 2008, Chepurnov et al. 2008, Poulickova & Mann 2008)). This type of study would permit clear definition of the life cycle strategies adopted by a large assemblage of species (Fig. 37), correlation of the dynamics of life cycle phases with physico-chemical variations in the water column, and identification of the environmental cues that trigger life cycle phase changes (e.g. temperature, irradiance levels, nutrient availability (macro- and microelements), parasite or predator pressure, prey availability, etc.) or promote the alternative development between the two phases (seasonality). Moreover, it would provide information on the prevalence of each life cycle phase throughout the year, which could give a clear indication of the rate of sexual turnover between life phases (sporadic, daily, monthly, yearly, or over longer time scales ?), which is essential for understanding population dynamics, species functioning and evolution.

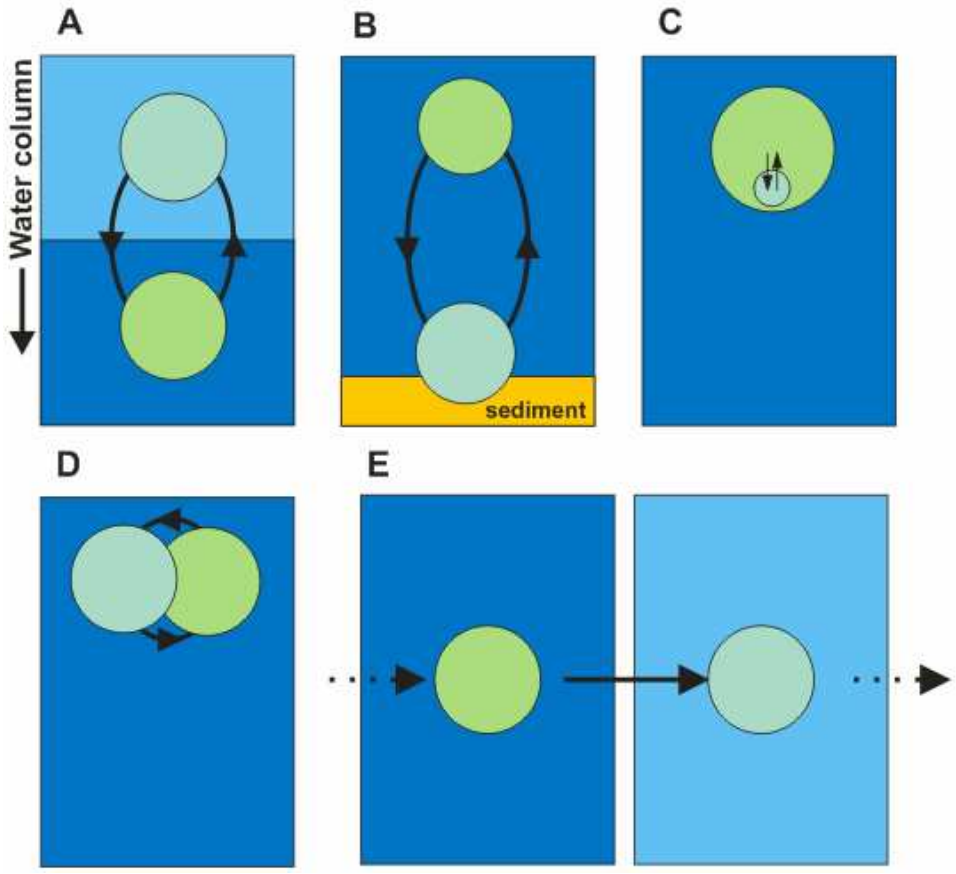


Figure 37. Putative models of life cycle and niche differentiation of the Calcihaptophycidae. A. Niche separation in space (different water masses) at the same time (e.g. *Helicosphaera carteri*, (Cros 2002)); B. Particular case of niche separation in space, with the haploid phase being benthic and the diploid pelagic (e.g. *Pleurochrysis pseudoroscoffensis*, based on (Gayral & Fresnel 1983)) ; C. Co-existence with dominance of one phase, as we suggested for *E. huxleyi*; D. Co-existence without dominance; E. Niche separation in time (seasonality) (e.g. *Coccolithus braarudii*, (Okada & McIntyre 1977)).

In parallel to these field studies, there is considerable scope for performing further complementary *in vitro* experiments in which the two life cycle phases of a wider range of species would be compared in terms of basic physiology, like growth rates and photosynthetic characteristics, but also responses to biotic and abiotic stresses. This approach will aid interpretation of observations made in nature and lead to a better understanding of life-cycle specific adaptations and optimal conditions for survival and growth. This would be particularly relevant in the context of predicting the responses of coccolithophores species (or communities) to future enviromental changes, including in terms of temperature, chemical composition of the atmosphere and pH variations of the water column (Rost & Riebesell 2004).

Furthermore, lab-based observations will be essential for unveiling new information about the processes of syngamy and meiosis. Attention should be focussed on elucidating the possible existence of mating types and determining whether cells can fuse with cells from the same clone (homothallism) or only with cells from a different clone (heterothallism). This would provide insights into how genes flow within and between populations. Presently, among the Calcihaptophycidae there are very few observations of cell fusion (*Pleurochrysis pseudoroscoffensis* (Gayral & Fresnel 1983) and *Coccolithus braarudii* (Houdan et al. 2004)) and meiosis (*Pleurochrysis pseudoroscoffensis* (Gayral & Fresnel 1983), *Calyptrophaera sphaeroideae* (Noel et al. 2004) *Coccolithus braarudii* and *Coronosphaera mediterranea* (Houdan et al. 2004)). Again, to our knowledge, crossing experiments between haploid strains have never been attempted and the factors that trigger induction of meiosis have never been clarified.

1.3. Origins of haplo-diplontic life cycles in haptophytes

The last theme studied during this thesis was the origin of the haplo-diplontic life cycle of haptophytes. We undertook preliminary assays to determine the ploidy level of cultured Pavlovophyceae strains (**Chapter 5**). From this work it was determined that one strain (RCC 1533) was diploid. This result, even though not definitively revealing the life cycle strategy present in this group, opens a range of perspectives to pursue research in this poorly known group. This strain can now be used for closer investigation of morphological and physiological properties. Moreover, during this analysis attention should be paid to the two different morphological states that we detected during our study (the flagellate and colonial forms). It is not very uncommon to observe some haploid cells in diploid Calcihaptophycidae culture strains (I. Probert, unpublished observations). If a haplo-diplontic life cycle is also a characteristic of the Pavlovophyceae, it will probably be possible to detect both ploidy states within some culture strains after careful analysis. It remains possible, however, that Pavlovophyceae exhibit complex life cycles, encompassing more than two forms, or instead that their life cycle is monophasic (diplontic) or even asexual, with cells multiplying solely by vegetative reproduction (mitosis). Haplo-diploidy has been reported in a single species of the Cryptophyta (Hill & Wetherbee 1986), which is the sister lineage of the Haptophyta. In this context, if the majority (or all) cryptophytes undergo haplo-diplontic life cycles like the prymnesiophytes, it is likely that the ancestral entity of these two lineages was also haplo-diplontic, and that among the modern Pavlovophyceae there will also be haplo-diplontic species.

Recently, molecular studies have been initiated on two other cultured Pavlovophyceae strains (Nosenko et al. 2007). These authors suggested that a full genome sequencing project of a pavlovophyte is warranted. The availability of this genetic information would, like for *E. huxleyi*, help to unveil much more about the hidden life cycles of these organisms and in so doing shed more light on the origins of haplo-diplonty in the haptophytes. For instance, searching for meiosis-related genes, as referred to above for *E. huxleyi*, may provide evidence for the ability to undergo meiosis, which would be indicative of an inherent sexual potential. This approach has been applied to several organisms in which sexuality had never been observed, for example in some diplomonad species from the genus *Giardia* (Ramesh et al. 2005, Malik et al. 2008, Schurko & Logsdon Jr. 2008).

1.4. Evolutionary considerations on the haptophytes haplo-diplontic life cycle

Despite the fact that the origin of the haplo-diplontic life cycle of haptophytes is still unresolved, it is clear that haplo-diploidy is widespread in the prymnesiophytes, being present in all non-calcifed and calcified groups and probably allowing the two phases of each species to exploit discrete ecological niches and so survive in unstable environments (Valero et al. 1992, Houdan et al. 2004). Moreover, heteromorphy appears to be common throughout the division, which probably reflects the adaptation of each phase to different conditions, facing different selective pressures.

This ubiquity is, however, rather surprising in light of the evolutionary history (more than 500 My), diversity (more than 350 described species) and breadth of ecological niches (coastal, oceanic, pelagic, benthic, brackish and freshwater) colonized by the modern taxa (Jordan & Chamberlain 1997, de Vargas et al. 2007). One might expect, *a priori*, to find some species that would have evolved from the primordial biphasic state towards a monophasic (haplontic or diplontic) life cycle (or asexuality), driven by some particular adaptation which would made them to reduce progressively one of the ploidy states. From the various examples presently available (e.g. *Coccolithus braarudii*, *Helicosphaera carteri*, *Coronosphaera mediterranea*) it generally appears that both life cycle phases can be present and form large populations in nature, giving a good indication of the maintenance of the haplo-diploid strategy rather then adaptation towards the other types of life strategies. However, present knowledge of the ecology of the majority of prymnesiophyte species is very limited and therefore in most cases it is difficult to recognize if there is a degree of dominance of one stage over the other.

A good model assemblage of species with which it is possible to make comparisons is the brown algae (Phaeophyta), which is a group of macroalgae (multicellular) that have been studied for centuries and for which in-depth knowledge on life cycles exists for several species (e.g. (Bell 1997, de Reviers 2002, Coelho et al. 2007)). In this group, the large majority of species are haplo-diplontic, but looking closer at each case, a high degree of variability is found between the two phases, e.g. cases in which the haploid individual is the larger, cases in which haploid and diploid individuals are of similar size, whether small or large. Moreover, some species, like those in the order Fucales, have clearly evolved a diplontic life cycle with the haploid phase reduced to gametes, like in metazoans. Had the brown algae been microscopic and/or uncommon, these differences may well have remained hidden. In the Haptophyta the same type of variability in life cycles might also exist, still hidden in the wide but barely explored extant diversity. In our study on *E. huxleyi*, we suggested that a certain degree of dominance of the diploid phase over the haploid might exist. This hypothesis would indicate that the apparently strict haplo-diplontic strategy with equality between phases may in fact be much more flexible, allowing species to adapt by favoring one of the ploidy phases. Moreover, heteromorphy can also be less dramatic than appears to be the case in the Calcihaptophycidae, as isomorphy or 'cryptic-heteromorphy' is common in the non-calcified taxa.

The Haptophta, as a group, consitute therefore an interesting monophyletic assemblage of organisms to test theories related to life cycle evolution and maintenance of haplo-diploidy. More studies and careful examination of a wide array of species are needed, both through field and laboratory research, the latter taking advantage of the large diversity of cultured strains presently available, notably in the Roscoff Culture Collection. Moreover, the recent generalization of genome sequencing projects and the future development of biomolecular tools to transform haptophyte species can clearly contribute significatively to push further the haptophytes as model organisms, with a wide interest for applied and fundamental research.

1.4. The COD-FISH method: further remarks

Examples of other applications

As final methodological remarks I would like to point out that beyond the wide applicability of the COD-FISH method to help resolving issues related to calcihaptophyte ecology and life cycles, it can evolve and be coupled with other techniques to explore other biological questions. For example, it is know that several coccolithophore species (or life cycle phases, like the haploid phase of *Coccolithus braarudii* (Parke & Adams 1960, Houdan

et al. 2006)) are mixotrophic, being able to perform photosynthesis but also to ingest by phagocytosis bacteria and probably other eukaryotic microbes. A few coccolithophore species appear to lack photosynthetic apparatus and so they may be operating as strict heterotrophs (Marchant & Thompson 1994). The joint utilization of predator and prey genetic probes can be used to unveil the mixotrophic abilities of some species and further recognize prey-type preferences, both in culture (Fig. 38) and in field samples.

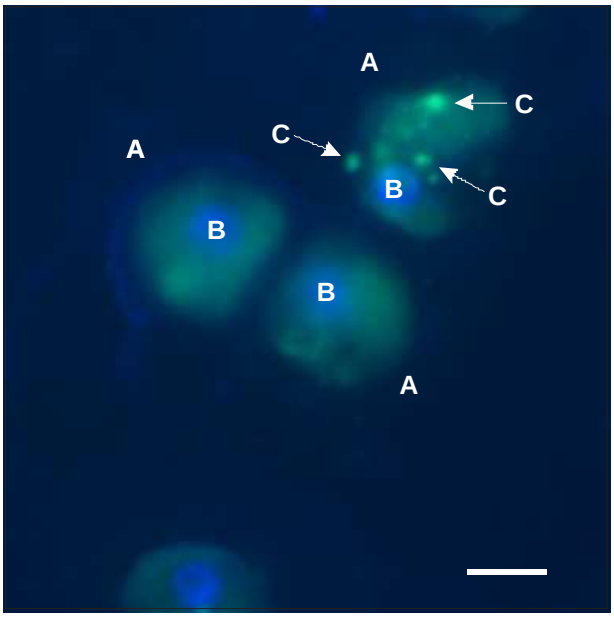


Figure 38. Cultured haploid cells of *Jomonlithus littoralis* (AC513) with ingested bacteria inside the cytoplasm detected by COD-FISH (preliminary assay performed in the frame of Daniella Mella-Flores master thesis, **Annex 4**). A. *J. littoralis* cells; B. *J. littoralis* nucleus stained with DAPI; C. Bacteria.

The same kind of technical concept could be used in the reverse sense, i.e. to discover which marine microbial predators prey on haptophytes, or it could be used to study symbiotic associations estabilished between haptophytes and other organisms (e.g. haploid cells of *Pleurochrysis scherffelii* have been observed as symbionts (or temporary symbionts) in a benthic foraminifera host (Hawkins & Lee 2001, Lee 2006)).

Recently, the application of FISH techniques (*sensu lato*) has been extended and associated with other methods that beyond simple genetic recognition of species, allow investigation of metabolic features of the targeted organisms. This method links FISH with autoradiography (MAR-FISH) and has been used mainly to study bacteria. Briefly, cultures or natural assemblages are incubated with a radiolabelled nutrient, like for example amino acids (or glucose, fatty-acids, phosphate, bicarbonate, etc.). Then samples are collected, hybridized with a probe for a given taxonomic group and finally revealed radiographically to see if the probed organisms utilized the radiolabelled substrate (Ouverney & Fuhrman 2000, Alonso &

Pernthaler 2005, Alonso-Sáez et al. 2008). This methodology will probably be soon tested with eukaryotes, including coccolithophores (coupled with the COD-FISH method).

Finally, repeating one of the concluding sentences from the article where we described this methodology, the COD-FISH method (**Chapter 2**) is not restricted to coccolithophores but can be applied to other microcalcifiers, such as the foraminifera or some calcareous dinoflagellates. Furthermore, the same morpho-genetic strategy can be applied to other protists for wish it may prove important to couple the genetic identity and the morphological features (e.g. (Stoeck et al. 2003, Kolodziej & Stoeck 2007)).

Limits and technical improvements

In general the COD-FISH technique is easily executed and is relatively rapid, since the results are acquired within approximatively 5-6 hours. However, it has some limitations that should be taken into account and improved in the future.

One limitation concerns the probe design and specificity testing. The quality of a probe is firstly limited by the quality of the 18S or 28S rDNA database that will used to search for the phylogenetic signature specific to the species (or group) that one wants to study. This database should be as complete as possible, containing not only sequences of the taxonomic group of interest, but also of a wide diversity of taxa. This will improve the specificity of a probe. Phylogenetic databases and various tools to design probes have been extensively improved in recent years, and are available to a much wider community of researchers interested in applying FISH related techniques (see: http://www.arb-silva.de/). After this step, probes should be tested against a relatively representaive diversity of cultured organisms, both to tune the optimal conditions of hybridization, that may vary slightly from probe to probe, but also to verify that the conditions used do not favour unspecific binding to other species. This testing step requires access to cultures, which may be a problematic issue for some groups of organisms or some research groups.

Another limitation that has to be taken into account mainly when dealing with species that are not very abundant, is the volume of water that is needed to be processed and the limitations of the fine pore size of the anodisc filters (Whatman, Maidstone, Kent, UK) that are used to collect the cells (these filters are used for COD-FISH because they are flat, they do not polarize the light, which is essential to visualize the coccoliths, and they resist all chemical treatments, particularly the ethanol treatments that damage other filter types). In my experience, over 800 ml of water sample, the filtration time is too long and destructive to the

organisms. Other filters or methods of water concentration should be developed and tested in the future in order to improve this part of the protocol.

Finally a limitation of this method when applied to large amounts of samples is the time spent for observation and analyses (identification and counting). This limitation, however, will be probably be improved in the near future with the advent of new motorized systems coupled to the microscopes that allow screening of samples and automatic recognition of shapes, forms or fluorescently labelled cells. Presently, this kind of technology has been used in several laboratories studying bacterial communities (e.g. Phillipe LeBaron, Banyuls-sur-Mer, CNRS France), but will soon be extended to other marine protists characterized by more complex shapes and large size ranges.

2. References

Alonso-Sáez L, Sánchez O, Gasol JM, Balagué V, Pedrós-Alio C (2008) Winter-to-summer changes in the composition and single-cell activity of near surface Arctic prokaryotes Environ Microbiol 10:2444-2454

Alonso C, Pernthaler J (2005) Incorporation of glucose under anoxic conditions by bacterioplankton from coastal north sea surface waters Appl Environ Microbiol 71:1709-1716

Amato A (2008) The sexual cycle of the diatom *Pseudo-Nitzschia*: from morphology through biology to genes. The Open University

Assmy P, Hernández-Becerril DU, Montresor M (2008) Morphologica Variability and Life Cycle Traits of the Type Species of the Diatom Genus *Chaetoceros*, *C. dichaeta*. Journal of Phycology 44:152-163

Bell G (1997) The evolution of the life cycle of brown seaweeds. Biological journal of the Linnean Society 60:21-38

Billard C (1994) Life Cycles. In: Green JC, B.S.C. Leadbeater e (eds) The Haptophyta Algae, Vol 51. Clarendon Press, Oxford, p 167-186

Bowers HA, Tengs T, Glasgow HB, Burkhoelder JM, Rublee PA, Oldach DW (2000) Development of rela-time PCR assay for rapid detection of *Pfiesteria piscicida* and related dinoflagellates. Appl Environ Microbiol 66:4641-4648

Bratbak G, Egge J, Heldal M (1993) Viral mortality of the marine alga *Emiliania huxelyi (*Haptophyceae) and the termination of the algal bloom. Marine Ecology Progess Series 93:39-48

Brownlee C, Taylor AR (2004) Calcification in coccolithophores. In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Carroll L (1865) Alice's adventures in wonderworld Vol. Signet Classics, New York, London

Castberg T, Larsen A, Sandaa RA, Brussard CPD, Egge JK, Heldal M, Thyrhaug R, van Hannen EJ, Bratbak G (2001) Microbial population dynamics and diversity during a bloom of the marine coccolithophorid Emiliania huxleyi (Haptophyta). Marine Ecology Progress Series 221:39-46

Chepurnov VA, Mann DG, Von Dassow P, Vanormelingen P, Gillard J, Inzé D, Sabbe K, Vyverman W (2008) In search of new tracable diatoms for experimental biology. BioEssays 30:692-702

Coelho SM, Peters AF, Charrier B, Roze D, Destombe C, Valero M, Cock JM (2007) Complex life cycles of multicellular eukaryotes: New approaches based on the use of model organisms. Gene 406:152-170

Coyne ET, Paul JH, Smith MC, Gray M (2004) Detection and quantification of red tide dinoflagellate *Karenia brevis* by real-time nucleic acid sequence-based amplification. Appl Environ Microbiol 70:4727-4732

Cros L (2002) Planktonic Coccolithophores of the NW Mediterranean. Universitat de Barcelona

Cros L, Kleijne A, Zeltner A, Billard C, Young JR (2000) New examples of holococcolith-heterococcolith combination coccospheres and their implications for coccolithophorid biology. Marine Micropaleontology 39:1-34

de Reviers B (2002) Biologie et phylogénie des algues, Vol Tome 1, Paris

de Vargas C, Aubry M-P, Probert I, Young J (2007) Origin and Evolution of Coccolithophores: From Coastal Hunters to Oceanic Farmers. In: Falkowski P, Knoll AH (eds) Evolution of Aquatic Photoautotrophs. Elsevier Academic Press, New York

de Vargas C, Probert I (2004) New keys to the Past: Current and future DNA studies in Coccolithophores. Micropaleontology 50:45-54

Delaroque N, Muller D, Bothe G, Pohl T, Knippers R, Boland W (2001) The complete DNA sequence of the Ectocarpus siliculosus virus EsV-1 genome. Virology 287:112-132

Edvardsen B, Eikrem W, Green JC, Andersen RA, Moon-van der Staay SY, Medlin L, K. (2000) Phylogentic reconstruction of the Haptophyta inferred from the 18S ribosomal DNA sequences and available morphological data. Phycologia 39:19-35

Evans C, Malin G, Mills GP, Wilson WH (2006) Viral Infection of *Emiliania huxleyi* (Prymnesiophyceae) leads to elevated production of Reactive Oxygen Species. J Phycol 42:1040-1047

Gayral P, Fresnel J (1983) Description, sexualité et cycle de développement d'une nouvelle Coccolithophoracée (Prymnesiophyceae): *Pleurochrysis pseudoroscoffensis* sp. nov. . Protistologica 19:245-261

Green JC, Course PA, Tarran GA (1996) The life-cycle of *Emiliania huxleyi*: A brief review and a study of relative ploidy levels analysed by flow cytometry. J Mar Syst 9:33-44

Harris EH (2001) *Chlamydomonas* as a model organism. Annual Review of Plant Physiology and Plant Molecular Biology 52:363-406

Hawkins EK, Lee JJ (2001) Architecture of athe Golgi apparatus of a scale forming alga: biogenesis and transport of scales. Protoplasma 216:387–395

Hill DRA, Wetherbee R (1986) *Proteomonas sulcata* gen. et sp. nov. (Cryptophyceae), a cryptomonad with two morphologically distinct and alternating forms. Phycologia 25:521-543

Houdan A, Billard C, Marie D, Not F, Saez A, Young G, Probert I (2004) Holococcolithophores-heterococcolithophores (Haptophyta) life cycles: flow cytometry analysis of relative ploidy levels. Systematics and Biodiversity 1:453-465

Houdan A, Probert I, Van Lenning K, Lefebvre S (2005) Comparison of photosynthetic responses in diploid and haploid life-cycle phases of *Emiliania huxleyi* (Prymnesiophyceae). Marine Ecology Progress Series

Houdan A, Probert I, Zatylny C, Véron B, Billard C (2006) Ecology of Oceanic Coccolithophores. I. Nutritional Preferences of the Two Stages in the Life Cycle of *Coccolithus braarudii* and *Calcidiscus leptoporus*. Aquatic Microbial Ecology 44:291-301

Jacquet S, Heldal M, Iglesias-Rodriguez D, Larsen A, Wilson W, Bratbak G (2002) Flow cytometric analysis of an Emiliana huxleyi bloom terminated by viral infection. Aquatic microbial ecology 27:111-124

Jordan RW, Chamberlain AHL (1997) Biodiversity among haptophyte algae. Biodiversity and Conservation 6:131-152

Kolodziej K, Stoeck T (2007) Cellular identification of a novel uncultured marine stramenopile (MAST-12 clade) small-subunit rRNAgene sequence from a norwegian estuary by use of fluorescence in situ hybridization-scanning electron microscopy. Appl Environ Microbiol 73:2718-2726

Laguna R, Romo J, Read BA, Wahlund TM (2001) Induction of Phase Variation Events in the Life Cycle of the Marine Coccolithophorid Emiliania huxleyi. Appl. Environ. Microbiol. 67:3824-3831

Lee JJ (2006) Algal symbiosis in larger foraminifera. Symbiosis 42:63-75

Lenski RE (1988) Dynamics of Interaction Between Bacteria and Virulent Bacteriophages. Advances In Microbial Ecology 10:1-44

Malik S-B, Pightling AW, Stefaniak LM, Schurko AM, Logsdon JM, Jr. (2008) An Expanded Inventory of Conserved Meiotic Genes Provides Evidence for Sex in Trichomonas vaginalis. PLoS ONE 3:e2879

Mann DG (1988) Why didn't Lund see sex in Asterionella? A discussion of the diatom life cycle in nature. In: F.E.Round (ed) Algae and the aquatic environment, p 385-412

Marchant HJ, Thompson LR (1994) Haptophytes in polar waters. In: Green JC, B.S.C. Leadbeater e (eds) The Haptophyta Algae, Vol Special volume No.51. The Systematics Association, Oxford

Martinez JM, Schroeder DC, Larsen A, Bratbak G, Wilson WH (2007) Molecular Dynamics of Emiliania huxleyi and Cooccurring Viruses during Two Separate Mesocosm Studies. Appl. Environ. Microbiol. 73:554-562

Mitchell DR (2000) *Chlamydomonas* flagella. Journal of Phycology 36:261-273

Muller DG (1977) Sexual reproduction in British *Ectocarpus siliculosus* (Phaeophyta). British Phycological Journal 12:131-136

Müller DGKH, Stache B, Lanka S (1990) A virus infection in the marine brown alga Ectocarpus siliculosus (Phaeophyceae). Botanica Acta 103:72-82

Nedelcu AM, Marcu O, Michod RE (2005) Sex as a response to oxidative stress: stress genes co-opted for sex. Proc. R. Soc. Lond. Biological Sciences 272:1935 - 1940

Noel M-H, Kawachi M, Inouye I (2004) Induced Dimorphic Life Cycle of a Coccolithophorid, *Calyptrosphaera sphaeroidea* (Prymnesiophyceae, Haptophyta). J Phycol 40:112-129

Nosenko T, Boese B, Battacharya D (2007) Pulsed-field gel electrophoresis analysis of genome size and structure in *Pavlova gyrans* and *Diacronema* sp. (Haptophyta). Journal of Phycology 43:763-767

Okada H, McIntyre A (1977) Modern coccolithophores of the Pacific and North Atlantic Oceans. Micropaleontology 23:1-55

Otto SP, Lenormand T (2002) Resolving the paradox of sex and recombination. Nature Reviews Genetics 3:252-261

Ouverney CC, Fuhrman JA (2000) Marine planktonic archaea take up amino acids Appl Environ Microbiol 66:4829-4833

Paasche E (2001) A review of the coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae), with particular reference to growth, coccolith formation, and calcificationphotosynthesis interactions. Phycologia 40:503-529

Parke M, Adams I (1960) The motile (*Crystallolithus hyalinus* Gaarder and Markali) and non-motile phases in the life-history of *Coccolithus pelagicus* (Wallich). Journal of the Marine Biological Association of the United Kingdom 39:263-274

Parke TG, Salas MF, Bolch CJ, Hallegraeff GM (2007) Development of a real-time PCR probe for quantification of the heterotrophic dinoflagellate *Cryptoperidiniopsis brodyi* (Dinophyceae) in environemntal samples. Appl Environ Microbiol 73:2552-2560

Penna A, Galluzzi L (2007) PCR techniques as diagnostic tools for the identification and enumeration of toxic marine phytoplankton species, Vol Proceedings of the NATO advanced study institute on sensor systems for biological threats:the algal toxins case. Springer, Pisa, Italy

Poulickova A, Mann DG (2008) Autogamous auxosporulation in Pinnularia nodosa (Bacillariophyceae) Journal of Phycology 44:215-231

Ramesh MA, Malik S-B, Logsdon Jr. JM (2005) A Phylogenomic Inventory of Meiotic Genes: Evidence for Sex in Giardia and an Early Eukaryotic Origin of Meiosis. Current Biology 15:185-191

Rost B, Riebesell U (2004) Coccolithophores Calcification and the Biological Pump: Reponse to Environmental Changes. In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Schurko AM, Logsdon Jr. JM (2008) Using a meiosis detection toolkit to investigate ancient asexual "scandals" and the evolution of sex. BioEssays 30:579-589

Simon N, Campbell L, Erla Örnlfsdttir E, Groben R, Guillou L, Lange M, Medlin LK (2000) Oligonucleotide Probes for the Identification of Three Algal Groups by Dot Blot and Fluorescent Whole-Cell Hybridization. The Journal of Eukaryotic Microbiology 47:76-84

Stoeck T, Fowle WH, Epstein SS (2003) Methodology of Protistan Discovery: from rRNA Detection to Quality Scanning Electron Microscope Images. Appl. Environ. Microbiol. 69:6856-6863

Valero M, Richerd S, Perrot V (1992) Evolution of Alternation of Haploid and Diploid Phases in Life Cycles. Trends in Ecology and Evolution 7:25-29

Van Valen L (1973) A new evolutionary law. Evolutionary Theory 1:1-30.

Wilson WH, Tarran GA, Schroeder D, Cox M, Oke J, Malin G (2002) Isolation of viruses responsible for the demise of an *Emiliania huxleyi* bloom in the English Channel. J. Mar. Biol. Ass. U.K. 82:369-377

Young JR, Geisen M, Probert I (2005) A Review of Selected Aspects of Coccolithophore Biology with Implications For Paleobiodiversity Estimation. Micropaleontology 51:267-288

Zhu F, Massana R, Not F, Marie D, Vaulot D (2005) Mapping of picoeucaryotes in marine ecosystems with quantitative PCR of the 18S rRNA gene. FEMS Microbiol Ecol 52:79-92

List of Publications

List of publications

Published

Frada, M., Not, F., Probert, I. and de Vargas, C., 2006. $CaCO_3$ Optical detection with fluorescent *in situ* hybridization: a new method to identify and quantify calcifying microorganisms from the oceans. Journal of Phycology, 42(6): 1162-1169.

Frada, M., Probert, I., Allen, M.J., Wilson, W.H. and De Vargas, C., 2008. The "Cheshire Cat" escape strategy of the coccolithophore *Emiliania huxleyi* in response to viral infection. Proceedings of the National Academy of Sciences USA, 105(41): 15944–15949.

In Press

Frada, M., Percopo, I., Young, J., Zingone, A., de Vargas, C., Probert, I., in press. First observations of heterococcolithophore-holococcolithophore life cycle combinations in the family Pontosphaeraceae (Calcihaptophycideae, Haptophyta). Marine Micropaleontology.

Submitted

Hui, L. Probert, I., Uitz, J., Claustre, H., Aris-Brosou, S., **Frada, M.**, Not, F., de Vargas, C. in prep. A newfound ancient diversity of protists dominates photosynthesis in open oceans. Proceedings of the National Academy of Sciences USA

In Preparation

Frada, M., Probert, I., Von Dassow, P. Wilson, W.H., Hinz, D., de Vargas. In prep. *In Situ* Survey of the calcified and non-calcified life cycle phases of the coccolithophore *Emiliania huxleyi* (Prymnesiophyceae). Aquatic Microbial Ecology

Frada, M., Young, J., Probert, I., Percopo, I., Zingone, A., Mella-Flores, D., Von Dassow' P., Lino, S., Martins, A., Parente, A., Cachão, M., de Vargas, C. in prep. Cross-Polarized Microscopy images from extant coccolithophores (Calcihaptophycidae , Haptophyta). Journal of Plankton Research

Mella-Flores, D., **Frada, M.**, Probert, I. de Vargas C. in prep. Competition between the haploid and diploid life cycle phases of the coccolithophore *Emiliania huxleyi*: an experimental approach.

ANNEX 1

Sex as an algal antiviral strategy

Peter J. Morin*

Department of Ecology, Evolution, and Natural Resources, 14 College Farm Road, Rutgers University, New Brunswick, NJ 08901

Emiliana huxleyi is a tiny eukaryotic alga that plays a huge role in the ecology and biogeochemistry of the world's oceans. Under favorable conditions it forms extensive blooms that are visible to mariners as milky-colored ocean waters, and that appear from space in LANDSAT images as chalky swirls that can cover thousands of square kilometers. The white color is caused by light scattered by numerous calcareous plates, called coccoliths, that armor the cell surface of *E. huxleyi* and give members of this group of algae their common name, the coccolithophores. The enormous amount of calcium carbonate sequestered in coccoliths during extensive algal blooms makes these algae important carbon pumps in the global carbon cycle. In this issue of PNAS, Frada *et al.* (1) describe a novel strategy used by *E. huxleyi* to evade viruses that contribute to the alga's boom-and-bust population cycles.

Algal-Viral Dynamics

Viruses play a major role in the dynamics of marine algal blooms, including the prominent ones formed by *E. huxleyi*. Recent estimates suggest that a large fraction of the primary productivity attributed to oceanic algae is lost to viruses that infect and kill algal cells (2). Other evidence suggests that viruses can cause much of the mortality of algal cells that ultimately causes the collapse of marine algal blooms (2). *E. huxleyi* is no exception to this, because it is known to be infected by a group of giant phycodnaviruses called EhVs (3). Given the intense selective pressure that EhVs should impose during the collapse of algal blooms, there should be strong selection for resistance to viral attack by the most abundant algae, and equally strong selection on viruses to circumvent algal resistance. Comparable rapid evolution of resistance to viruses is well known in laboratory studies of interacting populations of bacteria and phage (4). Such coevolutionary arms races, fancifully termed "Red Queen" dynamics by Lee Van Valen (5), form the core of traditional thinking about the evolutionary and population dynamics of predators and prey, diseases and hosts, and similar sets of species that act as exploiters and victims. The net result of Red Queen dynamics should be rapid evolution that simply maintains the ecological status quo. However, Frada *et al.* (1) show in their recent paper that there is another fascinating way for *E. huxleyi* to evade their viral enemies. This mechanism involves an induced transition from diploid bloom-forming cells to a morphologically distinct haploid life history stage that is apparently immune to some of the viruses that infect diploid cells.

E. huxleyi has a complex life cycle that alternates between nonmotile diploid coccolith-bearing cells that form extensive blooms, and motile flagellated haploid cells that are completely covered by organic scales but that lack coccoliths. Frada *et al.* (1) show that the haploid phase of the complex life cycle provides a refuge from viral attack, creating a resistant reservoir of haploid cells that can mate, repopulate the diploid phase of the life cycle, and presumably go on to create new blooms. Instead of rapidly evolving immunity to viral attack, as would be predicted by Red Queen dynamics, the production of haploid cells effectively makes *E. huxleyi* invisible to viral attack, leading Frada *et al.* to coin the term "Cheshire Cat dynamics" for the resulting decoupled interaction between haploid algae and viruses. Cheshire Cat dynamics have interesting implications for understanding the dynamics of boom-and-bust cycles in dominant oceanic algae, and also illustrate a previously unappreciated set of conditions that can select for the maintenance of sexual reproduction in organisms that remain capable of very rapid clonal population growth.

The production of haploid cells effectively makes *E. huxleyi* invisible to viral attack.

Red Queen Dynamics

Red Queen dynamics offer one of the more compelling explanations for the evolution and maintenance of sexual reproduction. Although many ideas have been proposed to explain the selective advantage conferred by sexual reproduction (6–8), much of the empirical evidence seems to support ideas that invoke the Red Queen. That evidence takes the form of correlations between the frequency of parasitism and sexual reproduction in populations that contain a mixture of sexually and asexually reproducing individuals (9), correlated spatial and temporal shifts in the genetic composition of parasites and their hosts (10, 11), and the potential for more rapid genetic change in sexually reproducing organisms (12, 13). Cheshire Cat dynamics provide a different and previously unrecognized advantage to sexual reproduction, as the induction of the haploid phase of the life cycle confers an invulnerable refuge against viruses that are known to attack the diploid phase of the life cycle. This advantage would also tend to oppose the disadvantage imposed by deleterious recessive alleles exposed to selection in the haploid phase of the life cycle. This purging of deleterious mutations has been proposed as another important consequence of sexual reproduction (14).

The results of Frada *et al.* (1) raise interesting questions about the dynamics of natural *E. huxleyi* blooms. One curious feature of *E. huxleyi* dynamics is that the same genetically distinct strains of diploid cells tend to recur in different blooms that form over time. This pattern is inconsistent with Red Queen dynamics, which would produce genetically different resistant strains over time, with each strain eventually being decimated by viruses that evolve ways to circumvent the resistance. However, if bouts of sexual reproduction reshuffle the combinations of genes in the *E. huxleyi* genome, how do the same recurrent dominant diploid strains reassemble from a pool of haploid gametes? One must imagine that an extraordinary number of diploid progeny are produced to reassemble those strains by chance, certainly possible given the enormous population sizes of microbes. Or, perhaps the recurrent genetic strains that consistently form blooms are cryptic species with rather restricted ranges of genetic variation and adaptation, despite the presence of sexual reproduction. Obviously, more study is needed to resolve the apparent contradiction presented by the genetic diversity that should be produced by sexual recombination and the

Author contributions: P.J.M. wrote the paper.

The author declares no conflict of interest.

See companion article on page 15944.

*E-mail: pjmorin@rci.rutgers.edu.

reduced genetic diversity in bloom-forming strains.

Another interesting problem concerns the factors that prevent bloom formation by the haploid phase of the life cycle. Frada *et al.* (1) challenged haploid and diploid algae only with viruses that have been isolated from diploid algae. This raises the question of whether haploid *E. huxleyi* may have their own, as yet unrecognized, set of viral pathogens that limit their ability to form blooms. Of course, it is also possible that blooms of haploid *E. huxleyi* occur but remain unrecognized because of the current limits of oceanographic sampling methods.

The mechanisms that confer viral resistance on the haploid phase of the *E. huxleyi* life cycle also remain uncertain. Frada *et al.* (1) suggest that the organic scales that closely cover the surface of haploid cells provide a possible mechanical defense against attachment by viral particles. As the authors point out, other examples of evolved resistance to viral attack usually involve changes in the composition of cell surface sites that promote attachment by viral particles. It is unclear how the simple meiotic transition from diploid to haploid would accomplish such a genetic change.

E. huxleyi is only one of a host of organisms that possess a complex life cycle that alternates between haploid and diploid phases. It will be fascinating to learn whether the advantages of Cheshire Cat dynamics are a general feature of most organisms with similar life cycles, or whether they are peculiar to *E. huxleyi*.

1. Frada M, *et al.* (2009) The ''Cheshire Cat'' escape strategy of the coccolithosphore *Emiliana huxleyi* in response to viral infection. *Proc Natl Acad Sci USA* 105:15944–15949.
2. Brussaard CPD (2004) Viral control of phytoplankton populations—A review. *J Eukaryot Microbiol* 51:125–138.
3. Wilson WH, *et al.* (2002) Isolation of viruses responsible for the demise of an *Emiliania huxleyi* bloom in the English Channel. *J Mar Biol Assoc UK* 82:369–377.
4. Chao L, Levin BR, Stewart FM (1977) A complex community in a simple habitat: An experimental study with bacteria and phage. *Ecology* 58:369–378.
5. Van Valen L (1973) A new evolutionary law. *Evol Theory* 1:1–30.
6. Williams GC (1975) *Sex and Evolution* (Princeton Univ Press, Princeton).
7. Maynard Smith J (1978) *The Evolution of Sex* (Cambridge Univ Press, Cambridge, UK).
8. Kondrashov AS (1988) Deleterious mutations and the evolution of sexual reproduction. *Nature* 336:435–440.
9. Lively CM (1987) Evidence from a New Zealand snail for the maintenance of sex by parasitism. *Nature* 328:519–521.
10. Dybdahl MF, Lively CM (1998) Host-parasite coevolution: evidence for rare advantage and time-lagged selection in a natural population. *Evolution (Lawrence, Kans)* 52:1057–1066.
11. Lively CM, Dybdahl MF (2000) Parasite adaptation to locally common host genotypes. *Nature* 405:679–681.
12. Colegrave N, Kaltz O, Bell G (2002) The ecology and genetics of fitness in *Chlamydomonas*. VIII. The dynamics of adaptation to novel environments after a single episode of sex. *Evolution (Lawrence, Kans)* 56:14–21.
13. Colegrave N (2002) Sex releases the speed limit on evolution. *Nature* 420:664–666.
14. Zeyl C, Bell G (1997) The advantage of sex in evolving yeast populations. *Nature* 388:465–468.

ANNEX 2

COD-FISH: a new method to detect and quantify calcifying microorganisms in open ocean

Detailed Protocol

Miguel Frada[1, 2*], Fabrice Not[2], Ian Probert[3], Colomban de Vargas[2]

[1] Centro de Geologia, Departamento de Geologia, Faculdade. de Ciências, Universidade de Lisboa, Edifício C6, 6.4.55, Campo Grande, 1749-016 Lisboa, Portugal
[2] Station Biologique, UMR7144 CNRS, INSU et Univeristé Pierre et Marie Curie, Place Georges Teissier, 29680 Roscoff, France
[3] Algobank-Caen Culture Collection, Université de Caen, 14032 Caen, France

* Corresponding author
Phone: 02 98 29 23 70
Email:frada@sb-roscoff.fr

COD-FISH background:

The Combined Calcareous Detection- Fluorescent *in situ* Hybridization (COD-FISH) is a technique developed to detect and quantify coccolithophore cells in the open ocean. The technique results from the association of: **(1)** A modified Tyramide Signal Amplification-Fluorescent *In Situ* Hybridization (TSA-FISH-(Schönhuber et al. 1997)) technique, initially developed for photosynthetic picoeukaryotes (Not et al. 2002). This first tool allows the taxonomic affiliation of coccolithophores species under epifluorescence microscopy. **(2)** Cross-polarized microscopy (Young et al. 1992), which allows the heterococcolith-bearing stage (diploid) identification of a coccolithophore life cycle. This is due to the optical properties of the heterococcoliths (calcareous scales that constitute the cell cover) that polarize the light. Thus, the heterococcolithophores will be identified and quantified by both epifluorescence and cross-polarized microscopies. In contrast, the holococcolithophores (cell-cover made of holococcoliths) or the non-calcified cells (both are the haploid stages, depending on the coccolithophore species, Billard 1994, Houdan et al. 2004) do not polarize the light with the same proficiency as the heteroccolith-bearing cells and can only be identified/quantified by epifluorescence microscopy. At the end of the COD-FISH protocol, the filters containing the cells can be retrieved for scanning electron microscopy analysis to achieve a more detailed study of the targeted specimens.

The main steps of the COD-FISH protocol are detailed below. Furthermore, a list of the main reagents, explanatory diagrams, and a list of valuable publications are provided at the end.

Protocol[1]:

I. Fixation

a. Fix the sample with 6mM Na_2CO_3~paraformadehyde 10% [reagent1] (10% PFA) (i.e. 1% final concentration). Mix gently.
b. Incubate the mix for 1 hour at 4ºC in the dark[2].

II. Filtration[3], Permeabilization and Gluing

a. After fixation, mix the sample gently then filter it through a 25mm, 0.22µm Anodisc filter (Whatman). Use a low vacuum pressure (c.a. 200mmHg).
b. Once the sample is filtered proceed to the ethanol bath series
    i. Add 2ml EtOH50%, incubate for 3min. Filter out.
    ii. Add 2ml EtOH80%, incubate for 3min. Filter out.
    iii. Add 2ml EtOH100% incubate for 3min. Filter out until the end.
    iv. Let the ethanol evaporate totally at room temperature (RT) in the filtration device.
    v. Add 1ml of 1% liquid gelatine[reagent2] prepared with 6mM Na_2CO_3[reagent3] (~30ºC) and incubate it for 2 min 30 sec to allow a good fixation of the cells onto the filter. Filter out until the end.
    vi. Remove the filter carefully from the filtration unit to a Petri slide and dry it at 35ºC.
    vii. Ideally store the Petri slide at -80 or -20ºC for long periods. The filter can be kept at RT in the dark during the period of usage.

III. In Situ Hybridization

III.1 Hybridization (3hours)

a. Prepare a "humid chamber" from a 50ml recipient (Fig.1). Insert a piece of Whatman paper (chromatography paper) and moisturize it with 700-800µl of hybridization buffer[reagent4] at appropriate stringency. Close the chamber and pre-warm it at 35ºC.
b. Cut the desired number of filter sample pieces with a scalpel and mark the upper face on the plastic border (e.g. with white out and black ink) (see Fig.2).
c. Prepare the hybridization mix (9vol. of hybridization buffer + 1vol. of HRP-probe + 1vol. of competing probes if necessary[4]).
d. Place the pieces of filter on a 6 well Teflon slide and add 10µl of the hybridization mix on top of the filter (6 filter pieces per slide).
e. Put the Teflon slide inside the pre-warmed humid chamber and incubate for 3hours at 35ºC in the dark.

---

[1] For further details on the theory of FISH look at Amann, et al. 1995
[2] Do not incubate longer than the recommended time to avoid cell loss.
[3] Do not let the filters dry between the first filtration and the 2 first ethanol baths.
[4] Nonspecific hybridization of a TSA-FISH probe to non-target sequences with a single mismatch to that probe may not be discriminated from specific target sequence hybridization regardless of stringency. The inclusion of an unlabeled complementary probe to these non-target sequences (competitor probe) should prevent hybridization of the TSA-FISH probe by competing for the target site.

III.2 Washing I (2 x 20min)

a. Prepare the washing buffer[reagent5] just before usage (Prepare 2x3ml per filter piece).
b. Distribute the buffer in two distinct 6 wells ELISA plates (Fig.3) and pre-warm them at 37ºC.
c. Transfer the filter pieces from the "hybridization chamber" to the corresponding well on the first ELISA plate[5] (be careful and plunge the filter to the bottom of the well)
d. Repeat the process twice for 20min each plate.

In the case of a simple hybridization with direct labelled probes (FITC or CY3), at the end of III.2, wash the filters in 6mM Na_2CO_3 (RT/ dark) and go to III.6.

III.3 Equilibration (15min)

a. Transfer the filter pieces from the washing buffer (2nd plate) to an equilibration buffer, previously distributed in an ELISA plate (TNT-1[reagent6]; it can be prepared previously and stored at RT).
b. Incubate for 15min at RT in the dark.

III.4 Enzymatic Reaction: signal amplification step (HRP~TSA-FITC) (40min)

a. Prepare the TSA mix solution[reagent7] (make it fresh). Vortex until the solution becomes homogeneous.
b. Depose 10µl of the solution on a new Teflon slide and transfer the filter from the TNT-1 buffer on top of the TSA mix solution.
c. Incubate in the dark for 40min at RT[6].

III.5 Washing II (2 x 20min + 10min)

a. Transfer the filter pieces to a pre-warmed TNT-2 buffer[reagent8] (Prepare 2x3ml per filter piece) in an ELISA plate. Incubate 2 times for 20min at 37ºC in the dark.
b. In a new ELISA plate, wash the filters for 10min in a 6mM Na_2CO_3 solution.

III.6 DAPI staining (5min + 10min + approx 10min)

a. Prepare the DAPI solution[reagent9]. Pipette 10µl of this solution on a new Teflon well and put the filter on top of it. Incubate at RT in the dark for 5min.
b. In a new ELISA plate, wash the filters in a 6mM Na_2CO_3 solution for 10min.
c. Dry the filter pieces at 35ºC.

IV. Microscopic Preparation and Observation

a. Pipette a couple of Microscopic oil[reagent10] drops onto each Teflon slide wells and put the filter on it.
b. Cover the filter with a cover-slip and seal it with regular nail varnish. Let it dry in the dark for few minutes.
c. Observations[7] can be made right away or the Teflon slide can be kept in

[5] To re-use the ELISA plates, submerge it in 1%HCl over-night and then rinse it in MilliO H_2O and dry.
[6] **ATTENTION**: Do not let the filter pieces dry during this step. This would create a strong fluorescent background under epifluorescence microscopy. If needed use a humid chamber with TNT-1 buffer.

the dark at 4ºC for later observation (for a maximum of 2 weeks without fluorescence lost).

V. Filters Recuperation for Scanning Electron Microscopy (SEM)

a. Remove carefully the cover-slip with a scalpel blade.
b. Wash the filters in TNT-2 buffer twice for 10min at 37ºC
c. Wash the filter pieces in 6mM Na_2CO_3 for 10min at RT.
d. Dry the filters pieces at 37ºC, and keep it dry at RT until SEM preparation.

[7] Use an epifluorescence microscope (excitation/emission filters were respectively 490/515 nm for FITC staining and 360/420 nm for DAPI staining). For each microscope field, and immediately after epifluorescence counting, coccolith-bearing cells can be detected and counted using visible light and cross-polarization, which is available on any basic Nomarski set up.

Reagents:

1- Na_2CO_3 6mM~Paraformaldehyde 10% (Vol. given for 100ml) **(storage:4ºC)**
PREPARE INSIDE A FUME HOOD BECAUSE THE PARAFORMALDEHYDE IS TOXIC

- Heat 50ml of Na_2CO_3 20mM + 15ml MilliQH_2O to 60ºC
- Add 10g of paraformaldehyde
- Add few drops (1-3) of 2M NaOH solution and stir rapidly until the solution has nearly clarified (should take 1-5min)
- Remove from heat source and add 33ml of PBS 3x
- Adjust pH to 7,5 with HCl (higher pH decrease efficiency of the fixative)
- Filtrate the solution through 0.2µm
- Quickly cool it down to 4ºC and store it for few weeks (2-3 max), or freeze it to -20ºC for longer preservation. Once thawed it has to be stored at 4ºC.

2- 1% Gelatine (Fluka, gelatin from porcine skin) ~ Na_2CO_3 6mM. **(Use at 35ºC; better to make it fresh in small volumes (e.g. 20ml) to avoid a long period of storage and the appearance of contaminants)**

3- Na_2CO_3 20mM (stock solution) [2g/1liter] **(storage: RT)**

4- Hybridization buffer (vol. given for 5ml) **(storage: -20ºC):**
PREPARE UNDER A FUME HOOD, THE FORMAMIDE IS TOXIC

• Na_2CO_3 20mM (6mM final concentration)	1,5ml
• NaCl 5M (0,9M final concentration)	900µl
• SDS 10%(0,01% final concentration)	5µl
• Blocking agent (10% final concentration)	495µl
• Tris-base 1M pH10,0 (20mM final concentration)	100µl
• Deionized Formamide (40% final concentration)	2ml

Formamide Deionization: Mix formamide during at least 1 hour with micro-beads (BioRad AG 501-x8(D) resin 20-50 mesh) in a proportion of 70:30 using an Erlenmeyer and a magnet (the beads are green and they become yellow). Filter everything with a coffee filter. Aliquot tubes of 1ml at 4ºC or -20ºC.

Blocking agent preparation (storage: -20ºC): Do not use the blocking agent belonging to the TSA-kits. It does not work properly. We recommend using ROCHE blocking for nucleic acid hybridization and detection (**AGENT CAT. / 1096176 50g**). For further information look to the reagent notice- www.roche-applied.com/pack-insert/1096176a.pdf)

5- Washing buffer (for 60ml) **(storage: make fresh)**

• Na2CO3 20mM (6mM final concentration)	18ml
• MilliQ H_2O	39,648ml
• SDS 1%	600µl
• NaCl 5M (0,037M f final concentration)	552µl
• Tris-base 1M pH 10,0 (20mM f final concentration)	1,2ml

Table: Concentrations of NaCl in washing buffer (37ºC) at different concentrations of formamide in hybridization buffer (35ºC) (Pernthaler et al. 2001)

% formamide in hybridization buffer	mM NaCl in washing buffer
20	145
25	105
30	74
35	52
40	37
45	26
50	19
55	13
60	9
65	8
70	5

6- Equilibration TNT-1 buffer (for 250ml) **(storage: RT)**

- Na_2CO_3 20mM (10mM final concentration) 125ml
- MilliQ H_2O 92,3ml
- Tween 20 (to stabilize the HRP) 185µl
- NaCl 5M (0,037M final concentration) 7,5ml
- Tris-base1M, pH9,0(20mM final concentration) 25ml

7- TSA mix reaction solution **(storage: make fresh, components stored at 4ºC)**

- Amplification Diluent 1vol.
  (Amplification diluents are included in the Fluorescein tyramide reagent pack)
- Dextran Sulfate 40% 1vol.
- Fluorescein Tyramide (FT) 0,04vol.

Ex. To 220µl, mix 110µl of AD and 110µl of DS and 4.4µl of fluorochrome solution

Fluorescein tyramide reagent pack, Renaissance catalog No. SAT701; contents: Fluorescein tyramide for 100-300 slides + amplification diluent 1x30ml.

8- TNT-2 buffer (to 250ml) **(storage: RT)**

- Na_2CO_3 20mM (6mM final concentration) 75ml
- MilliQ H_2O 142,3ml
- Tween 20 (HRP stabilizer) 185µl
- NaCl 5M (0,037M final concentration) 7,5ml
- Tris-base 1M pH10,0 (20mM final concentration) 25ml

9- DAPI solution (200µl) **(storage: 4ºC)**

- Na_2CO_3 20mM 199µl
- DAPI 1mg/ml (5µg/ml final concentration) 1µl

10- IMMERSION OIL for microscopy (ordinary use/ non- fluorescence) nd= 1,516 23ºC OLYMPUS 04 Japan.

Explicative diagrams

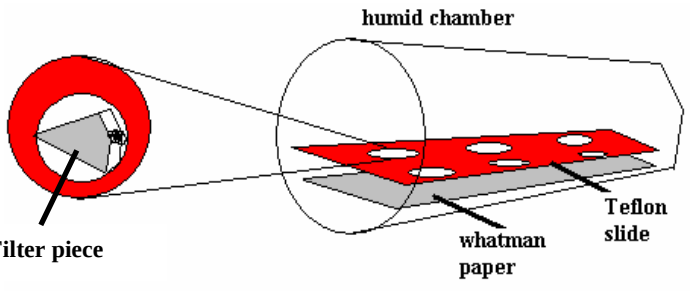


Figure 1. Humid chamber scheme.

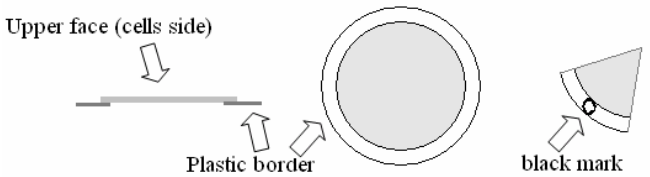


Figure 2. Anodics filter scheme and markage for COD-FISH utilization.

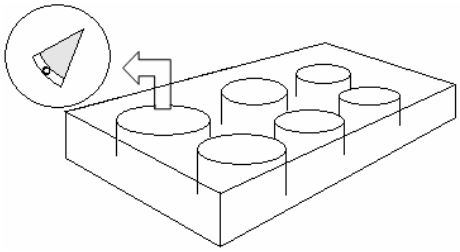


Figure 3. ELISA plate with an Anodisc filter at the bottom of a well.

Selected Bibliography

Amann, R. I., Ludwig, W. &Schleifer, K.-H. 1995. Phylogenetic Identification and In Situ Detection of Individual Microbial Cells Without Cultivation. *Microbiological Reviews.* 59:143-69

Billard, C. 1994. Life Cycles. *In* J.C. Green and B.S.C. Leadbeater, e. [Eds.] *The Haptophyta Algae*. Clarendon Press, Oxford, pp. 167-86

Houdan, A., Billard, C., Marie, D., Not, F., Saez, A., Young, G. &Probert, I. 2004. Holococcolithophores-heterococcolithophores (Haptophyta) life cycles: flow cytometry analysis of relative ploidy levels. *Systematics and Biodiversity*. 1:453-65

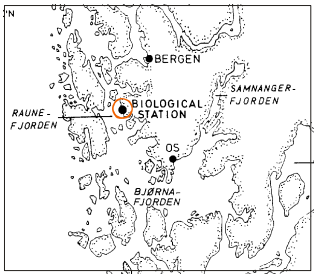
Not, F., Simon, N., Biegala, I. C. &Vaulot, D. 2002. Application of Fluorescent In Situ Hybridization Coupled With Tyramide Signal Amplification (FISH-TSA) to Assess Eukaryotic Picoplankton Composition. *Aquatic Microbial Ecology*. 28:157-66

Schönhuber, W., Fuchs, B., Juretschko, S. &Amann, R. 1997. Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. *Appl. Environ. Microbiol.* 63:3268-73

Young, J. R., Didymus, J. M., Bown, P. R., Prins, B. &Mann, S. 1992. Crystal assembly and phylogenetic evolution in heterococcoliths. *Nature*. 356

ANNEX 3

Title: Mesocosm 2008, COD-FISH and flow cytometry data
(part of this information is included in Chapter 2.2)



A. COD-FISH analyses data

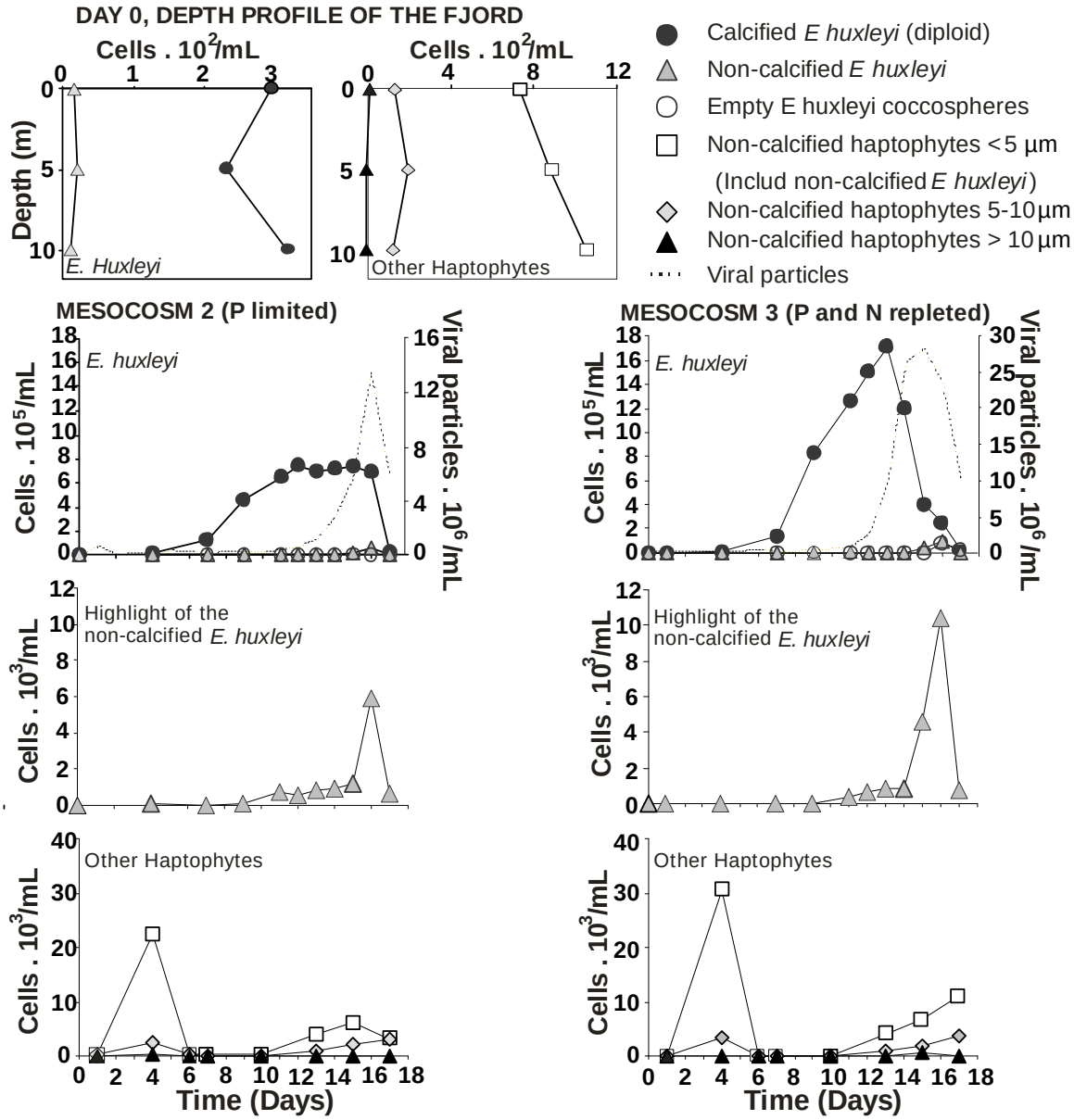


Figure 1. COD-FISH analysis (A) of the **c**alcified and non- calcified haptophytes comunity throughout the mesocosm 2008 experiment.
Two mesocosm enclosures are represented (bag 2- left and bag 3- right).
Emiliania huxleyi was analysed with the EG28-03 probes and the total haptophytes with the probe Prym-02 (see Chapters 2.1 and 2.2).

Title: (cont.) Mesocosm 2008, COD-FISH and flow cytometry data (some of this information is part of the Chapter 2.2)

B. Flow cytometry analyses data

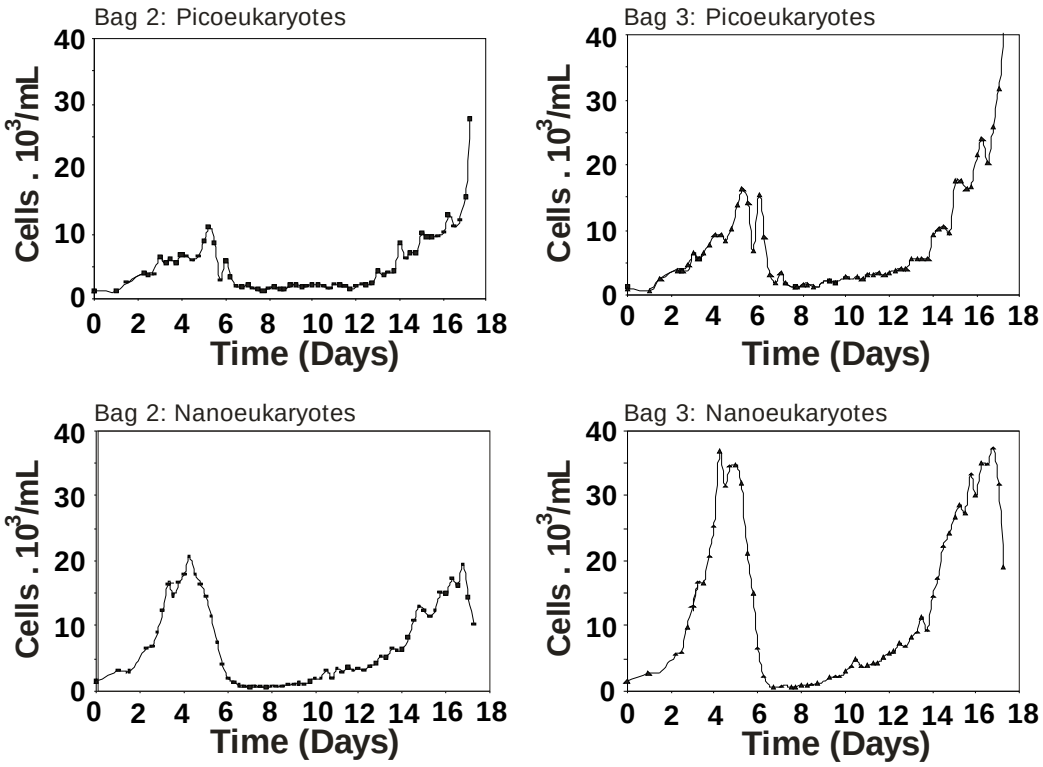


Figure 2. Flow cytometric analysis (B) of the total eukaryotic community.
Picoeukaryotes and nanoeukaryotic (including non-calcified haptophytes) community are represented.

Title: Vertical distribution of haptophytes and other Eukaryotes in Villefranche-sur-mer (France)

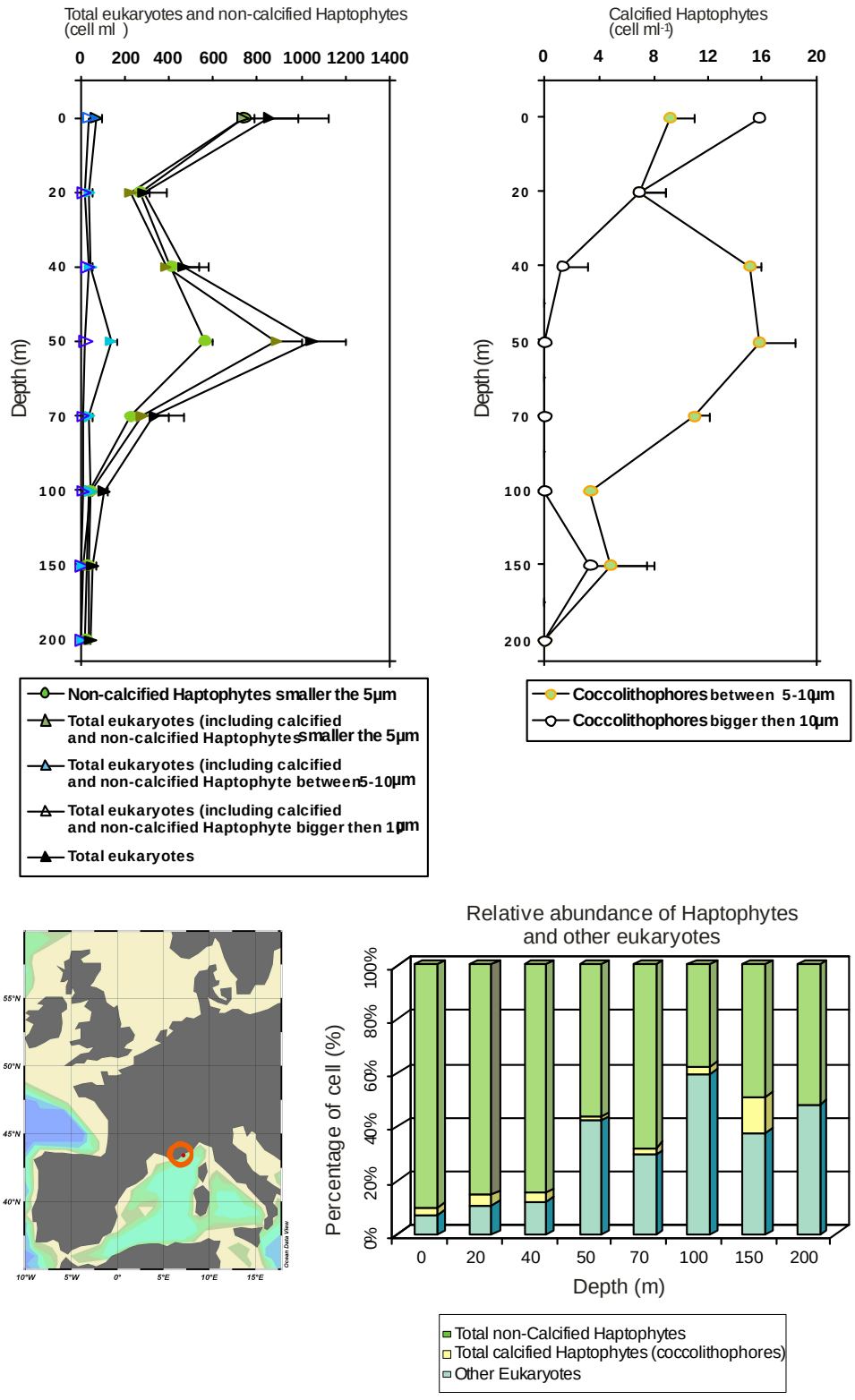
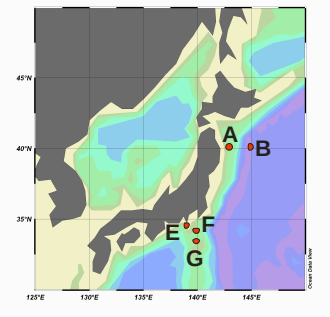


Figure 3. Abundance of calcified and non.calcified haptophytes and other indiscriminated eukaryotes collected at various depth in the Point C' (43°41'10 N 7°18'00 E) in Villefranche-sur-mer (France) the 12th September 2007 during the BOOMEDEX experiment.
The analysis of the haptophytes was performed by COD-FISH with the Prym-02 probe.

Title: Vertical distribution of haptophytes along a cruise in west Pacific ocean
(part of this information is included in Chapter 2.2)



Station	Coordinates
A	40°N; 143°E
B	40°N; 145°E
E	34°N; 140°E
F	34°N; 139°E
G	33°N; 140°E

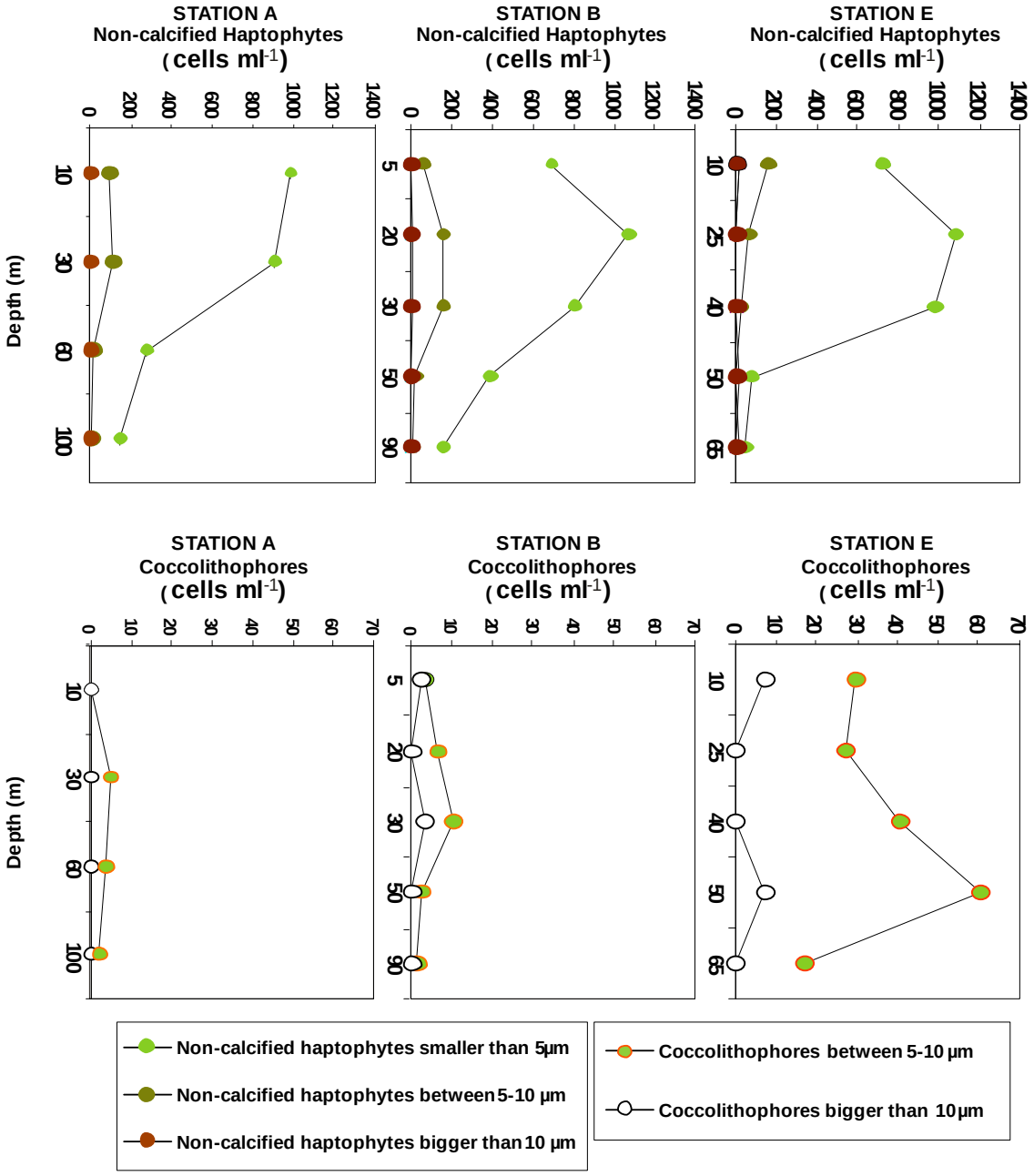


Figure 4. Abundance of calcified and non-calcified collected at various depth in the west Pacofoc close to the Japanese east coast, during May and June 2007.
The analysis of the haptophytes was performed by COD-FISH with the Prym-02 probe.

Title: (cont.) Vertical distribution of haptophytes along a cruise in west Pacific ocean

(part of this information is included in Chapter 2.2)

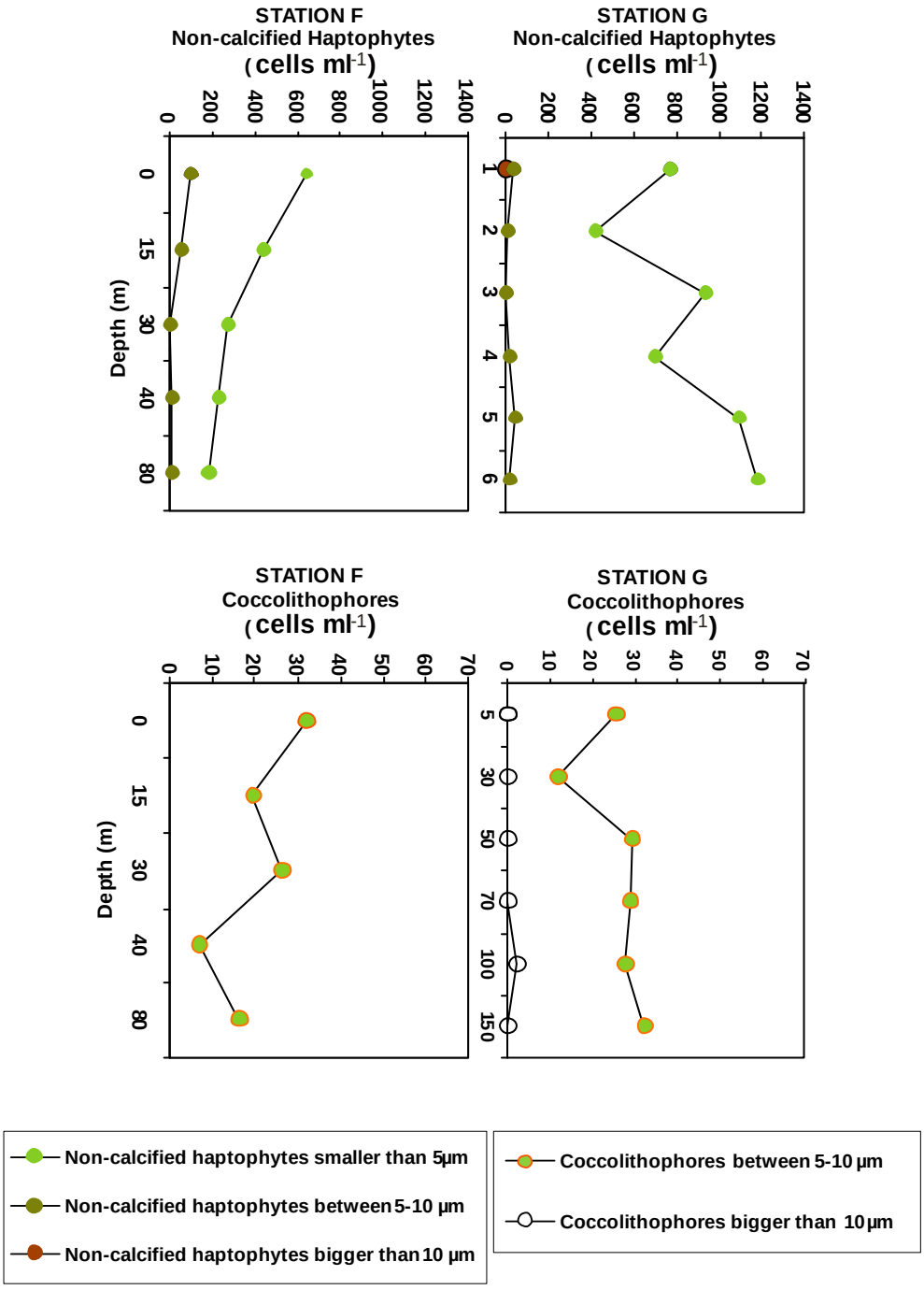


Figure 5. Abundance of calcified and non-calcified collected at various depth in the west Pacofoc close to the Japanese east coast, during May and June 2007 (continuation).
The analysis of the haptophytes was performed by COD-FISH with the Prym-02 probe.

Title: seasonal distribution of haptophytes in coastal environment off-shore Roscoff
(part of this information is included in Chapter 2.2)

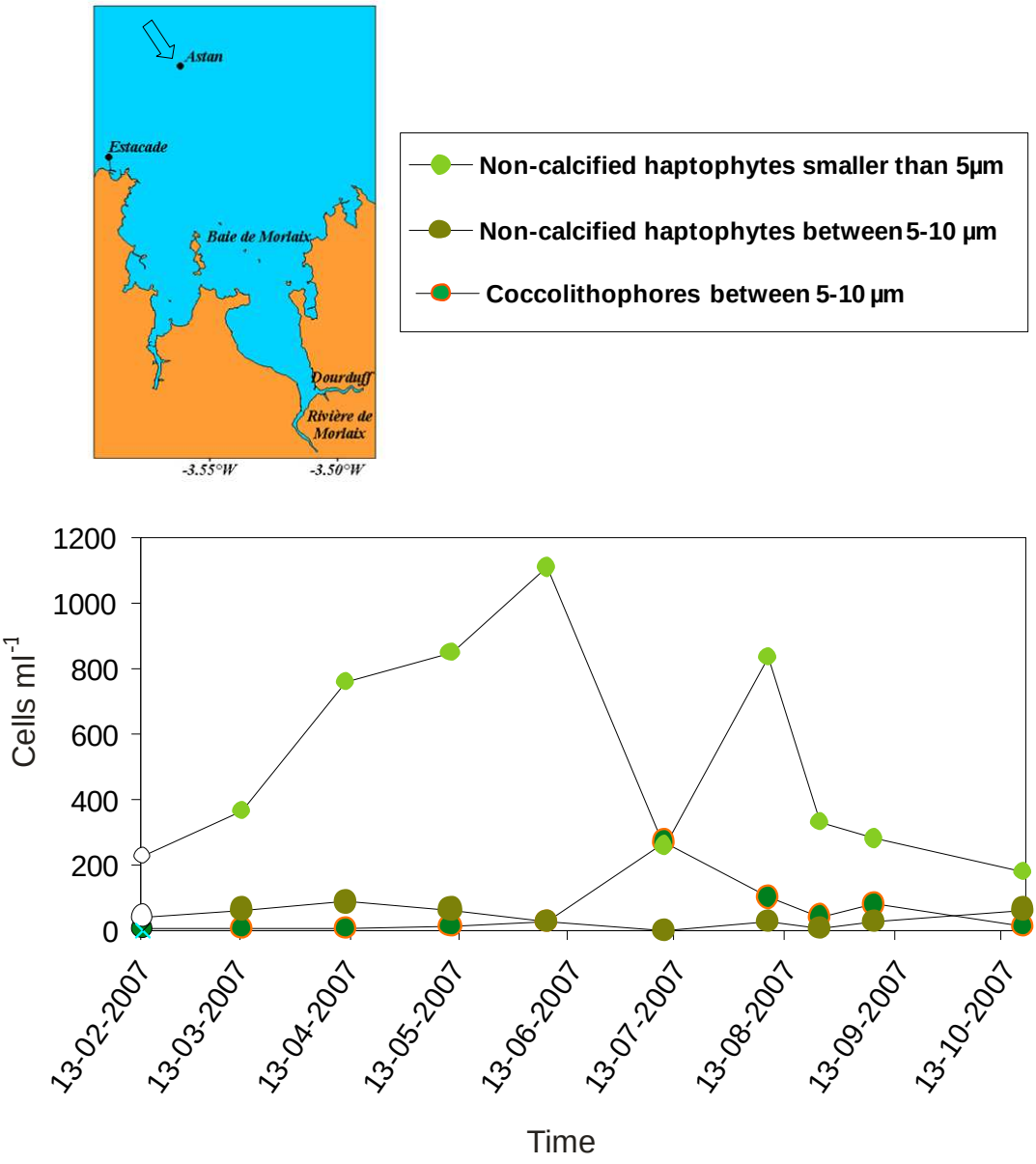
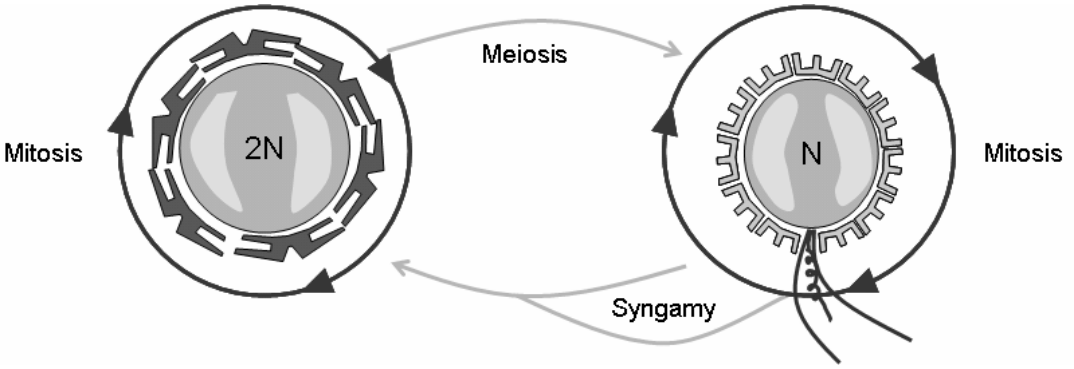


Figure 6. Seasonal distributin of calcified and non-calcified haptophytes haptophytes algae during 2007 in the SOMLIT-ASTAN (48°46′N, 3°57′W) station off-shore Roscoff (France).
The analysis of the haptophytes was performed by COD-FISH with the Prym-02 probe.

ANNEX 4

Master de Sciences et Technologie
« Mention Sciences de l'Univers, Environnement et Ecologie »
« Spécialité Océanographie et environnements marins »

«Biologie comparative des cycles de vie chez les coccolithophores»



Daniella MELLA-FLORES

Mémoire de Master
Effectué sous la co-direction de Miguel Frada et Colomban de Vargas
Equipe EPPO, Plancton Océanique
Année universitaire 2006/2007

Sommaire

I. Introduction

I.1. Généralités

Les océans, couvrant les deux-tiers de la surface de la terre, sont en grande partie dominés par les organismes unicellulaires, en particulier plusieurs phyla de protistes. Parmi eux, les coccolithophores, les diatomées et les dinoflagellés sont les groupes fonctionnels dominants depuis les dernières 200 millions d'années. Ils sont aujourd'hui parmi les plus importants acteurs dans la production primaire, en étant notamment à la base de la chaîne alimentaire marine. Ces organismes jouent un rôle fondamental dans le cycle biogéochimique de divers éléments dont le carbone (C), l'oxygène (O), l'azote (N), le soufre (S) et le fer (Fe) et ils sont des acteurs clés dans les échanges océan-atmosphère et dans l'exportation de ces éléments vers la lithosphère. Ces actions influent ainsi de façon décisive sur l'environnement et sur l'équilibre de l'écosystème à une échelle globale (Falkowski et al., 2004).

I.2. Le modèle d'étude: Les coccolithophores

Les coccolithophores, avec environ 280 morpho-espèces décrites, sont le groupe majeur du phylum Haptophyta (Jordan et Chamberlain, 1997). Elles comprennent toutes les haptophytes potentiellement capables de produire des écailles calcifiantes (coccolites) dans au moins un stade de leur cycle de vie. Elles forment un groupe monophylétique dans la classe des Prymnesiophyceae (Edvardsen et al., 2000), récemment nommée "Calcihaptophycidae" (De Vargas et Probert, in press).

Le groupe des coccolithophores est le plus souvent considéré comme un groupe de microorganismes océaniques préférant des eaux oligotrophes[1], c'est en effet là qu'on en trouve la plus grande diversité. Elles sont néanmoins susceptibles de coloniser tous les types des milieux aquatiques dans des proportions plus ou moins importantes (Billard et Inouye, 2004). Ils sont en effet présent dans la plupart des océans et des mers du globe, avec toutefois une diversité nettement plus élevée en région tempérée et sub-tropicale (Okada et McIntyre, 1979).

I.2.1. Caractéristiques générales des coccolithophores

Les coccolithophores sont le plus souvent mobiles, au moins pendant une partie de leur cycle de vie. Elles possèdent généralement, comme toutes les Prymnesiophyceae, deux flagelles égaux à subégaux et lisses (Green et Hori, 1994), (Fig.1).

[1] Oligotrophe : Milieu marin pauvre en nutriments

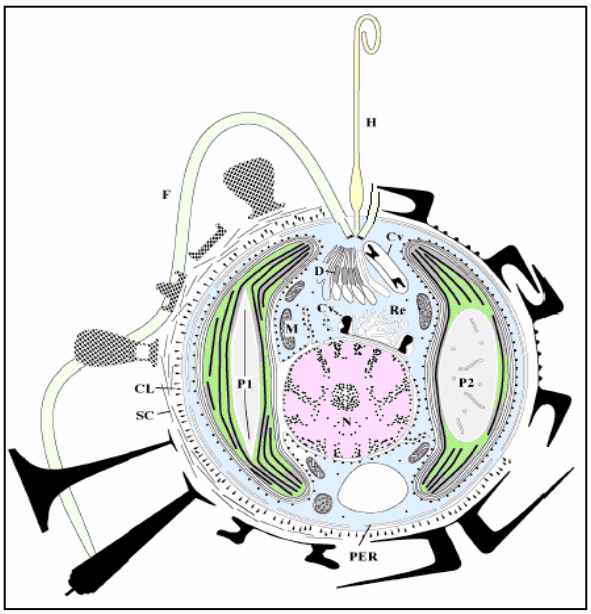


Figure 1. Schéma de la structure d'une coccolithophore. **H**- haptonème; **F**- 2 flagelles (seulement un représenté dans la figure); **C**- coccolites qui entourent la cellule et qui forment la coccosphère ; **B**- couche d'écailles organiques. **B** et **C** sont produits intracellulairement. La cytologie interne ressemble en générale à celle d'un organisme photosynthétique typique (d'après Billard and Inouye 2004).

Deux des caractéristiques les plus remarquables chez ces organismes sont les écailles calcaires qui recouvrent la cellule (les coccolithes) et la présence d'un appendice particulier : l'haptonème. Les coccolites sont des structures inorganiques en forme d'écailles de carbonate de calcium ($CaCO_3$), produites intracellulairement dans l'appareil de Golgi et transférés à l'extérieur de la membrane par un traffic de vésicules. Une fois imbriqués autour de la cellule ils forment la coccosphère. Plusieurs hypothèses existent sur la fonction de l'armure de coccolithes (Young, 1994) :protection, flotation, régulation par la lumière et rôle métabolique (source de carbone pour la photosynthèse ou pour éliminer l'excès de Ca^{2+} du cytoplasme) (Billard, 1994). L'haptonème, unique aux haptophytes, est situé entre les deux flagelles mais il peut être absent dans certaines espèces (par exemple *Emiliania huxleyi*) ou dans certains stades de leur cycle de vie. Cet appendice pourrait intervenir dans la détection des obstacles, dans la capture des agglomérats et le transport des proies, et dans l'adhérence aux sédiments (Inouye et Kawachi, 1994).

I.2.2. Rôle dans l'écosystème

D'un point de vue biogéochimique les coccolithophores jouent un rôle primordial dans la régulation de la chimie du carbone et dans ses échanges entre l'atmosphère, les eaux

océaniques de surface, et les sédiments profonds (De Vargas et Probert, in press). Ainsi les espèces qui forment des efflorescences (voir chapitre suivant), seraient responsables de la moitié de toute la précipitation actuelle du $CaCO_3$ dans les océans. En effet, à la mort de l'algue, la coccosphère tombe vers le fond des océans ; les coccolites se dissocient et s'accumulent pour constituer le composant majeur des sédiments carbonatés profonds (Fig. 2) (Paasche, 2001)et (Honjo, 1996.).

D'autre part quelques espèces d'haptophytes, comme *E.huxleyi* et *Phaeocystis sp.,* sont également de gros producteurs de diméthylsulfure (DMS). Le DMS joue un rôle central dans le cycle biogéochimique global du soufre. Dans l'atmosphère, les produits d'oxydation du DMS altèrent le budget radiatif de la planète *via* la formation d'aérosols de sulfate (Fig. 2) (Charlson et al., 1987) et (Andreae et Crutzen, 1997). Ils seraient ainsi en partie responsable des pluies acides et favoriseraient la nucléation des nuages, dispersant la lumière du soleil et affectant, en conséquence, le climat à une échelle globale (Rost et al., 2003) et (Malin et Steinke, 2004).

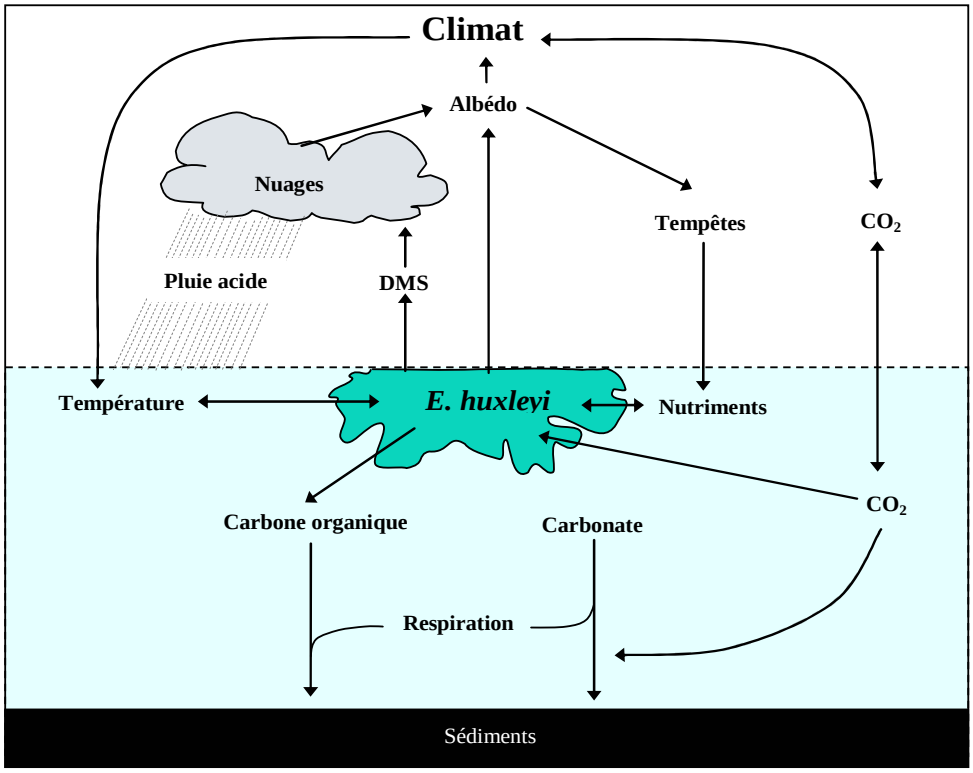


Figure 2. Rôle d'*Emiliana huxleyi* dans les échanges biogéochimiques
Le DMS produit par E. huxleyi est libéré dans l'athmosphère et conduit à la formation d'aérosols de sulfate. Ces aérosols refroidissent le climat en constituant la principale source de noyaux de condensation des nuages (NCN) au-dessus des océans. Une forte densité de NCN augmente l'albédo ou la réflectivité des nuages, ce qui augmente la quantité de radiations solaires reflétées vers l'espace.
A la mort cellulaire, la sédimentation des coccolithes alimente les sédiments crayeux et donc le stock de carbone des fonds marins.

I.2.3. Le cycle de vie chez les coccolithophores

L'ensemble des données actuelles semble montrer que les coccolithophores, et les haptophytes en général, sont caractérisés par un cycle de vie haplo-diploïde (Billard et Inouye, 2004) où les deux stades ont des morphologies différentes (hétéromorphe), (voir annexe 1). La découverte de cette alternance entre deux stades de ploïdie et de morphologie différente a été réalisée d'une part, par l'observation de la transition spontanée d'une phase à l'autre chez des cellules en culture, et d'autre part, par la découverte d'un stade intermédiaire possédant des caractéristiques des deux phases. Plus récemment des études moléculaires combinant phylogénie et estimation de la taille des génomes ont confirmé ces observations (Houdan et al., 2004.).

Malgré l'attention considérable portée aux coccolithophores, et notamment à l'espèce ubiquiste *Emiliania huxleyi*, un certain nombre de questions fondamentales au sujet de leur biologie de base demeurent sans réponse. Un des problèmes les plus importants porte sur la signification écologique et évolutive des cycles de vie haplo-diploïdes hétéromorphes dans ce groupe d'organismes (Houdan et al., 2004.). De fait, à ce jour, presque toutes les recherches sur l'écophysiologie d'*E. huxleyi* ont été conduites sur la phase diploïde (2N) calcifiante, capable de produire des efflorescences couvrant des larges aires dans la surface de la colonne d'eau (Fig. 3) ((Holligan et al., 1993), (Brown et Yoder, 1994),(Tyrrell et Merico, 2004). En revanche, la physiologie de la phase haploïde (N) non-calcifiante demeure inconnue.

Plusieurs études récentes ont démontré que la terminaison des blooms d'*E.huxleyi* 2N est due en grand partie à l'infection lytique des cellules 2N par des virus spécifiques, les coccolithovirus, qui déciment en masse les populations ((Jacquet et al., 2002), (Castberg et al., 2001), (Bratbak et al., 1993), (Brussaard, 2004)). Jacquet et Castberg ont aussi constaté qu'à la suite de l'infection virale, une population caractérisée par les mêmes propriétés photosynthétiques que les cellules d'*E. huxleyi* 2N mais avec un phénotype différent apparaît dans le milieu. Cette nouvelle population de cellules, apparemment résistante aux virus, colonise la colonne d'eau.

Récemment, une étude mené par Frada *et al.* (soumise) a démontré que le stade N d'*E.huxleyi* est résistant aux coccolithovirus capables d'infecter et tuer le stade 2N. De plus, cette étude a vérifié *in vitro* que les cellules N ont la possibilité de se développer et former des populations denses seulement après la chute des cellules 2N par l'attaque de virus (Fig. 4). Les virus pourraient ainsi être une force biologique clef contrôlant le cycle haplo-diploïde chez *E. huxleyi*. Or très peu d'études ont été réalisées sur la réponse des deux stades du cycle

de vie des coccolithophores (N et 2N) aux conditions environnementales essentielles telles que la lumière, la turbulence, et la source, composition et concentration des nutriments.

Figure 3 Les « Blooms » d'*Emiliania huxleyi.* *Emiliania huxleyi* (Haptophyta, Coccosphaerales) est le coccolithophore le plus abondant et ubiquiste dans les océans actuels. Lorsque cette micro algue trouve des conditions favorables de croissance, sa multiplication sur de vastes étendues océaniques produit des marées turquoises suffisamment importantes pour être visibles de l'espace Holligan *et al.*, 1993. Cette coloration provient de la réflexion de la lumière par les cellules recouvertes en carbonate de calcium. On parle alors de blooms, ou de floraisons phytoplanctoniques.

En 2005 Houdan *et al.* publient un premier travail qui compare la réponse photosynthétique des deux stades du cycle de vie chez *E. huxleyi* (Houdan et al., 2005). Cette étude montre que l'état 2N n'est pas soumis à photoinhibition[2], tandis que l'état N montre une réponse photoinhibitrice semblable à celle de la plupart des espèces de microalgues.

Chez deux autres espèces de coccolithophores, *Coccolithus braarudii* and *Calcidiscus leptoporus*, ces mêmes auteurs suggèrent que la phase N est plus sensible à la turbulence et plus adaptée aux milieux pauvres. Chez *C. braarudii*, la phase N présente également un comportement mixotrophique[3] (Houdan et al., 2006). Cette caractéristique est présente chez plusieurs haptophytes flagellées autre que les coccolithophores, tel que *Chrysochromulina*

[2] Photoinhibition : Stade physiologique de stress qui a lieu chez les organismes photosynthétiques soumis à des intensités lumineuses excessives.

[3] Mixotrophie : Même si la mixotrophie peut comprendre différents formes de nutrition (voir définitions dans Jones, 1994 ; Holen & Boraas, 1995), ici on va se référer aux organismes capables d'acquérir de l'énergie et/ou des nutriments à la fois par autotrophie phototrophique (en utilisant l'énergie lumineuse et nutriments inorganiques) et par hétérotrophie phagotrophique (en ingérant des bactéries ou des particules pour postérieur digestion et utilisation des dérivés organiques)

ssp. et *Prymnesium* ssp. (Jones et al., 1994). La mixotrophie serait peut-être maintenue dans le stade haploïde où la coccosphère est plus souple, voire absente, ce qui faciliterait la prédation et la phagocytose de particules (Houdan et al., 2006). En revanche, l'état 2N semble être plus adapté à des conditions turbulentes et il est plus compétitif dans des milieux riches en nutriments.

Ces résultats apportent une première idée sur les rôles potentiellement différents des deux stades de cycle de vie dans l'écosystème ; ils ouvrent aussi de nouvelles perspectives pour des études comparatives basées sur d'autres facteurs écologiques, aussi bien abiotiques que biotiques.

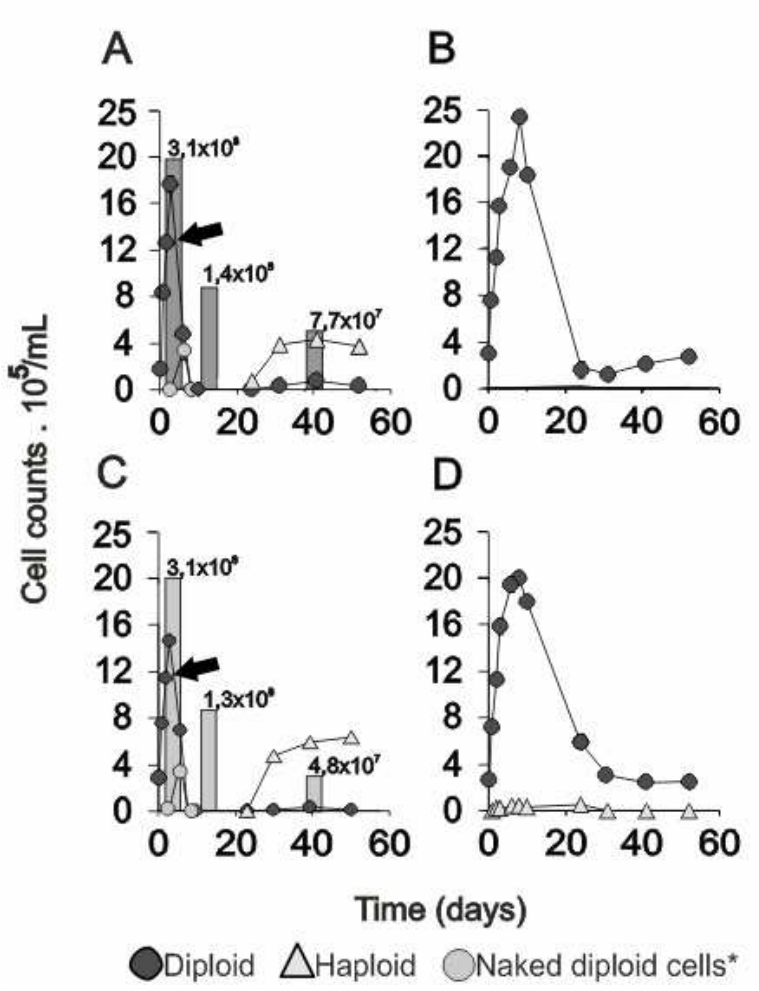


Figure 4. Infection virale de différents stades du cycle de vie (N et 2N) d'*E. huxelyi*.
Les courbes représentent les différentes populations des coccolithophores (haploïde et diploïde) et les barres les concentrations virales. La flèche indique le jour d'infection (en A et C seulement).Dans cette expérience menée par Frada *et al.* (sous presse) on peut voir qu'en **A** les cellules diploïdes infectées sont tuées en quelques jours par rapport aux cellules non infectées (en **B**). La diminution du nombre de ces dernières est due à la déplétion de nutriments après de plus de 20 jours en culture. Dans des situations de mélanges de cellules haploïdes et diploïdes (**C**), lorsque les cellules 2N sont tuées par des virus, les cellules N commencent à coloniser le milieu et deviennent dominantes. Au contraire lorsqu'il n'y a pas de virus dans le milieu (**D**), les cellules 2N restent performantes et les cellules N restent à de très faibles densités.

I.3. But du travail

Dans le cadre de la thèse de Miguel Frada (Equipe *EPPO* – Evolution du Plancton et PaléoOcéans, UMR-7144), consacrée à l'étude des cycles de vie chez les coccolithophores, et à la suite des observations effectués pendant son travail, des questions importants concernant la physiologie différentielle entre les deux phases du cycle de vie d'*E.huxleyi* ont été mises en évidence:

A- - Plusieurs questions concernent l'interaction et la succession entre les deux phases du cycle de vie d'*E. huxleyi* avant, pendant et après une infection virale.

Pourquoi est-ce la phase diploïde 2N qui forme des efflorescences, et pourquoi la phase haploïde N se développe-t-elle seulement après le déclin des 2N causé par l'attaque virale?

1) La phase diploïde a-t-elle un avantage physiologique (par ex. acquisition de nutriments) par rapport à la phase haploïde ?
2) La phase diploïde contrôle-t-elle le développement de la phase haploïde au moyen de molécules extracellulaires (infochimie)?

Quelles sont les implications écologiques et évolutives de cet avantage des diploïdes par rapport aux haploïdes ?

B- D'autres questions concernent le développement des deux phases du cycle de vie d'*E. huxleyi* à des intensités lumineuses différentes.

Récemment Houdan a montré que les cellules N sont photo-inhibées sous l'effet d'intensités lumineuses élevées (supérieures à 400-500 µE) (Houdan et al., 2005). Cette observation suggère une séparation de niche entre les cellules haploïdes et diploïdes, où des milieux plus lumineux favorisent la phase diploïde. Cependant, aucune étude n'a encore montré le développement de populations haploïde et diploïde dans des conditions lumineuses différentes. D'autres questions surviennent.

1) Comment les deux phases du cycle de vie d'*E.huxelyi* se développent sous différents intensités lumineuses? Quel est l'effet sur la croissance et la physiologie des cellules ?

1) Quelles sont les implications écologiques de ces réponses ?

C- Enfin Houdan (Houdan et al., 2006) a suggéré que les cellules haploïde de *C. braarudïï* pourraient être mixotrophes. La mixotrophie est bien connue chez quelques espèces primitives

d'haptophytes. Chez les coccolithophores cependant, l'existence de cette caractéristique a été observée seulement pour le stade haploïde de l'espèce *C.braarudii*. Dans cette espèce les cellules haploïdes peuvent à la fois phagocyter des bactéries et faire de la photosynthèse. En revanche le stade diploïde est exclusivement autotrophe. Tel observation, a amène une nouvelle perspective sur le rôle du stade haploïde et a augmenté le spectre d'adaptations possibles et de niches écologiques exploités par cette espèce.

La mixotrophie est donc très mal connue chez les coccolithophores.

1) La plupart des espèces sont-elles mixotrophes ?
2) La mixotrophie est-elle associée au stade haploïde, ou bien les stades diploïdes peuvent-ils aussi être mixotrophes ?

En résumé, ce travail a pour but général d'étudier l'écophysiologie différentielle entre les deux phases du cycle de vie d'*Emiliania huxleyi* en détail, et d'autres espèces de coccolithophores plus généralement. *E. huxleyi* est une des espèces les plus importantes du phytoplancton océanique en terme d'écologie globale (calcification, pompe biologique, échange de gaz ocean-atmosphère) ; il est donc crucial d'interpréter sa physiologie intégrale, c'est-à-dire dans l'ensemble de son cycle de vie. Cette étude apporte des données originales concernant la physiologie et l'écologie de la face cachée des coccolithophores : leur phase haploïde. Elle permet d'émettre de nouvelles hypothèses et d'apprécier les interactions entre cycles de vie, écosystème, et cycles biogéochimiques.

II Matériels et Méthodes

II.1. Matériel biologique

Toutes les souches utilisées dans ce travail proviennentde la Collection de Cultures de Microalgues de l'Université de Caen Basse-Normandie.

Nous nous sommes particulièrement concentrés sur une souche d'*Emiliania huxleyi* (AC472) originaire de la côte Ouest de Nouvelle Zélande. Les cellules de cette souche ont été d'abord isolées dans la phase 2N, puis la souche a présenté un changement de phase (méioses) au cours de sa troisième année de culture. Cette souche a donc par la suite été cultivée sous ses deux phases. Comme les phases N et 2N utilisés dans nos expériences sont très proches génétiquement, nous avons un très bon outil pour faire des comparaisons entre les deux stades du cycle de vie.

Un résumé avec les caractéristiques de la souche d'*E. huxleyi* et les autres espèces utilisées se trouve dans la Table 1.

Tableau 1. Souches utilisées (http://www.unicaen.fr/algobank/)

Espèce	Descripteur	Souche		Isolé par	Date isolement	Origine
		Caen	Autre			
Emiliania huxleyi	(Lohman) Hay et Mohler	AC472	TQ26	I.Probert	1998	Nouvelle Zélande
Schyphosphaera apstenii	*	AC504	TW15	I.Probert	2001	Espagne
Jomonlithus sp.	**	AC513	Esp5		2002	Espagne
Prymnesium parvum	Nanton	AC36	HAP36	C. Billard	1981	France
Calyptrosphaera sphaeroidea	*	AC507	UTEX-LB1940	D. Klaveness	1973	Norvège
Crysochromulina camelle	Leadbeater	AC56	HAP56	J. Fresnel	1985	France
Pleurochrysis carterae	Beech and Wetherbee	AC11	HAP11	H. Lepailleur	1965	France

* : Pas décrite ** : Probert, I., in prep.

II.2. Milieu de culture

Le milieu utilisé pendant toutes les expériences a été le **Milieu K** (Keller et al., 1987). Pour le préparer, de l'eau de mer prélevée au large de Roscoff est vieillie pendant un à deux et filtrée sur filtre 0,2 µm à l'aide d'un système Millipore (Rippka et al., 2000). Ensuite, des vitamines et des sels nutritifs sont ajoutés (annexe 2). Finalement le milieu est stérilisé par filtration sous hotte à flux laminaire et ensuite stocké à la température d'expérimentation. Pour les expériences avec milieu K dilué (K/2, K/10), les dilutions on été faites avec de l'eau de mer filtrée.

II.3. Conditions de culture et échantillonnages

II.3.1. Expérience 1 : Compétition entre stades du cycle de vie

Pour étudier la compétition intra-spécifique entre la phase N et 2N d'*E. huxleyi* des expériences de substitution ont été réalisées (Harper, 1977). Des cultures en tripliquat ont été faites dans des flacons de polycarbonate de 200 mL (Nalgène) dans 5 combinaisons différentes : 100%N, 75%N-25%2N, 50%N-50%2N, 25%N-75%2N et 100%2N. La concentration cellulaire initiale dans toutes les situations a été de $1x10^3$ cellules/mL. Les expériences ont été réalisées sur deux concentrations différentes de milieux afin d'évaluer

l'effet des nutriments sur la compétition (K/2, considéré comme milieu riche et K/10, considéré comme milieu pauvre). L'expérience a été réalisée en cycle jour/nuit (12/12 heures) et l'éclairement a été fourni par des tubes fluorescents (Sylvania Daylight, 58W/154). Pour cette expérience, l'éclairement a été fixé à 100 $\mu E.m^{-2}.s^{-1}$ et la température a été maintenue à 20°C. Les cultures ont été acclimatées dans ces conditions pendant deux semaines avant commencer l'expérience.

Afin d'étudier la croissance cellulaire par cytométrie en flux, une aliquote de 1 mL de culture a été prélevé chaque jour à la même heure pendant toute la durée de l'expérience. Une aliquote de 2 mL a également été prélevée chaque jour les mesures de pH.

II.3.2. Expérience 2 : Consommation des sels nutritifs

Afin d'étudier l'efficacité dans la consommation de l'azote et du phosphore des deux phases du cycle de vie d'*E. huxleyi*, des cultures en tripliquat des stades N et 2N ont été faites dans 1 L pendant 6 jours. Les conditions d'éclairement et de température ont été les mêmes que pour l'expérience précédente. Comme pour l'expérience antérieure, des aliquotes de 1 mL ont été prélevés tous les jours pour suivre la croissance cellulaire.

Pendant la phase exponentielle de croissance, des aliquotes de 100 mL ont été prélevés afin de mesurer les nitrates (NO_3^-), nitrites (NO_2^-) et phosphates (PO_4^{2-}). Ces aliquotes ont été filtrés à travers un filtre GF/F 25 mm afin d'éliminer les cellules qui pourraient interférer avec les analyses. Les échantillons filtrés ont été mis dans des flacons en polyéthylène (HDPE, type nalgène) lavés à l'acide Chlorhydrique 10% et rincés à l'eau miliQ et puis conservés à -20°C jusqu'à l'analyse.

Deux prélèvements en tripliquat ont été faites aussi pour la mesure de l'azote ammoniacal (NH_4), un le jour 2 et l'autre le jour 4 de croissance exponentielle. Ces aliquotes ont été également filtrés et versés sur des flacons autoclavables Fisherbrand. Immédiatement après le prélèvement les réactifs sont ajoutés à l'aide de dispensettes dans l'ordre suivant : Réactif 1, bouché et agité par renversement, Réactif 2 bouché et agité par renversement. Les flacons fermés ont été placés dans l'obscurité et à température ambiante 12 heures au minimum (passage dans les 2 à 3 jours suivant l'ajout des réactifs).

Les taux de consommation de sels nutritifs ont été calculés comme la pente de la concentration du nutriment dans le milieu versus le temps.

II.3.3. Expérience 3 : Evaluation des facteurs de répression/inhibition potentiels entre les phases du cycle de vie.

Afin d'évaluer un possible effet de répression/inhibition d'une phase du cycle sur l'autre, des expériences de changement de milieu ont été faites. Pour ceux-ci, les milieux de culture obtenus à la fin de la phase exponentielle de l'expérience 3 ont été récupérés et divisés en deux. Le milieu récupéré a été d'abord enrichi avec des sels nutritifs pour arriver à une concentration de K/10 (on a supposé un épuisement total des sels nutritifs pendant la phase exponentielle). Les expériences ont été faites en tripliquat. La procédure est résumée dans la figure 5.

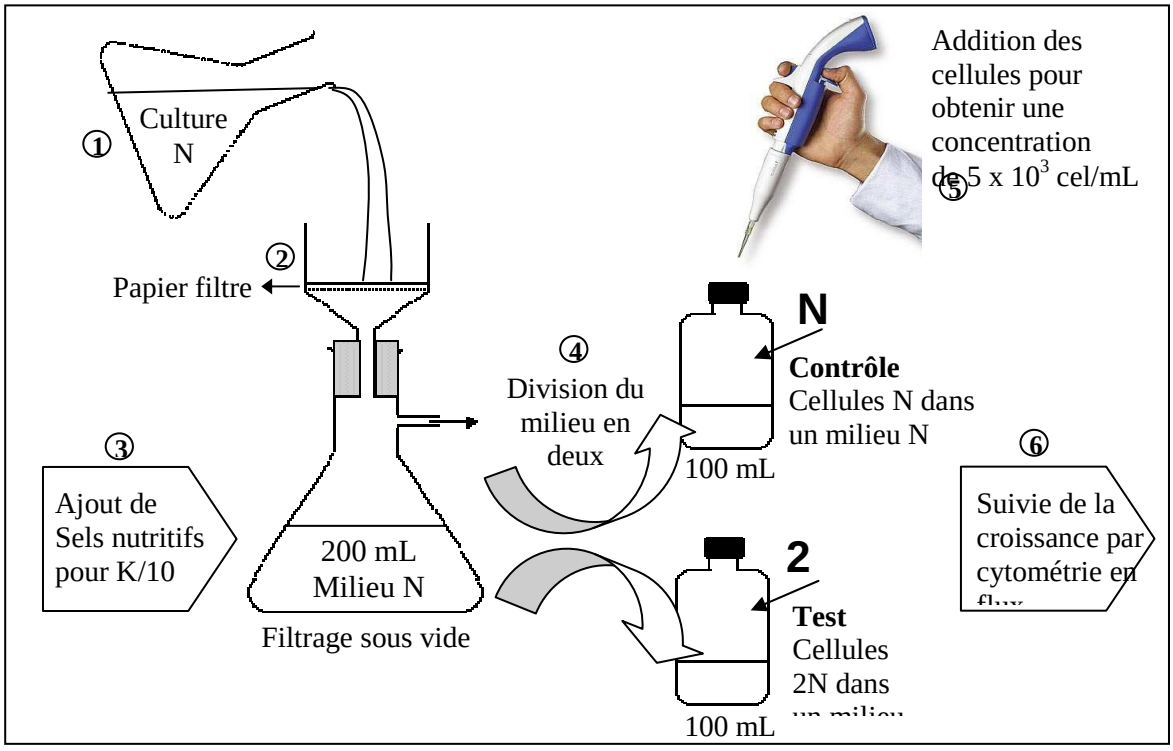


Figure.5 : Evaluation des potentiels facteurs de répression/inhibition entre les stades N et 2N *d'Emiliania huxleyi*.La figure montre un schéma de la méthodologie réalisée lors du changement du milieu de culture en prenant l'exemple du milieu obtenu à partir d'une culture de cellules haploïdes (N). La même procédure a été réalisée pour le milieu obtenu à partir d'une culture diploïde (2N).

II.3.4. Expérience 4 : Influence de l'intensité lumineuse

Des cultures (en 5 répliquats) des phases N et 2N d'*E.huxleyi*, préalablement acclimatés pendant trois semaines, ont été faites dans 3 intensités lumineuses différentes : Basse lumière (BL : 15 $\mu E.m^{-2}.s^{-1}$), moyenne lumière (ML : 100 $\mu E.m^{-2}.s^{-1}$) et forte lumière (FL : 300 $\mu E\ m^{-2}.s^{1}$) pendant 17 jours. Les cultures ont été faites sur milieu K/10 dans des flacons de polycarbonate de 200 mL (Nalgène). La concentration cellulaire initiale dans tous les traitements a été de $5x10^2$ cellules/mL. La température a oscillé autour de 22°C pendant

l'expérience, avec un minimum de 20°C dans les cultures à BL et un maximum de 24°C dans les cultures à FL.

Afin de quantifier la densité cellulaire, une aliquote de 1 mL de culture a été prélevée chaque jour à la même heure pendant toute la durée de l'expérience pour les analyses cytométriques. Une aliquote de 2 mL a également été prélevée chaque jour pour l'analyse de l'activité photosynthétique à travers le PAM.

II.3.5. Expérience 5 : La mixotrophie chez les haptophytes

La capacité de broutage a été testée dans différentes espèces de haptophytes (calcifiantes et non calcifiantes). Des cultures *d'Emiliania huxleyi* (N et 2N), *Schyphosphaera apstenii* (ploïdie inconnue), *Jomonlithus* sp. (N et 2N), *Prymnesium parvum* (ploïdie inconnue), *Calyptrosphaera sphaeroidea* (ploïdie inconnue- probablement N), *Crysochromulina camelle* (ploïdie inconnue) et *Pleurochrysis carterae* (2N) ont été cultivés dans un milieu K/2 pendant 1 mois afin de réduire les nutriments disponibles et d'induire (favoriser, provoquer) le broutage des bactéries présentes dans les cultures (les cultures utilisés ne sont pas axéniques). Après cette période, 1mL de chaque culture a été prélevé, fixé avec du para-formaldéhyde 1% et filtres sur un filtre anodisc de 0,2µm. Une fois sur le filtre, les cellules ont été déshydratées par une séries de bains d'éthanol (50, 80 et 100%) et finalement recouvertes avec de la gélatine 1% (méthodologie de préparation des filtres basé sur (Frada et al., 2006). Les bactéries ont été après ciblées par une « méthodologie d'hybridation de fluorescence in situ » : CARD-FISH- (Alonso-Saez et al., in press) (Fig.6).

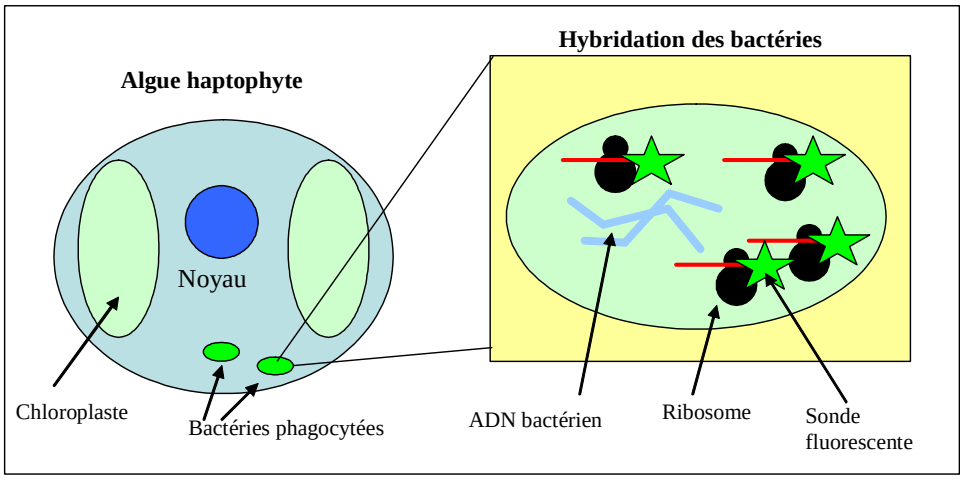


Figure 6 : principe de la technique de FISH (hybridation in situ fluorescent avec amplification du signal grâce à la tyramide) utilisé pour détecter des algues haptophytes ayant réalisé de broutage. Les cellules fixées et perméabilisées sont mises en présence de sondes de type oligonucléotidique marquées par l'enzyme horseradish peroxydase (HRP). Après hybridation des sondes aux sites cibles (ribosome), le substrat (tyramide-fluorescéine) est ajouté à la préparation. La réaction du substrat avec l'HRP provoque un signal fluorescent qui permet alors la localisation des cellules bactériennes. Les cellules peuvent être observées par microscopie à épifluorescence.

II.4. Méthodes d'analyse utilisées

II.4.1. Cytométrie en flux

L'appareil utilisé est un cytomètre FACsort (Becton Dickinson). Un volume déterminé d'échantillon est injecté à vitesse constante dans une gaine liquide sous pression. Les cellules passent alors une à une devant le faisceau laser (excitation à 488 nm). Deux types de paramètres sont recueillis : la diffusion de la lumière due à la particule et la fluorescence. Les diffusions dans l'axe du laser (Forward Light Angle Scatter ou FALS) et perpendiculairement à l'axe (Right Angle Scatter ou RALS) dépendent de la taille et de l'indice de réfraction des particules. De plus, une série de filtres optiques permet de séparer les différents signaux de fluorescence et permet donc leur mesure simultanée. Ainsi, la fluorescence des pigments photosynthétiques (Chlorophylle *a* et Phycoérythrine) et celle des marqueurs fluorescents spécifiques d'un constituant cellulaire, comme l'ADN, peuvent être détectées. Des microbilles fluorescentes calibrées de 0,95 µm (10^5 mL^{-1}, Polysciences) ajoutées à chaque échantillon servent à normaliser les différents paramètres et à vérifier l'alignement de l'appareil. Le cytomètre en flux permet ainsi de déterminer la concentration cellulaire d'une culture en calibrant précisément le débit du liquide de gaine et en mesurant la quantité d'évènements par seconde pendant une durée déterminée (Fig.7).

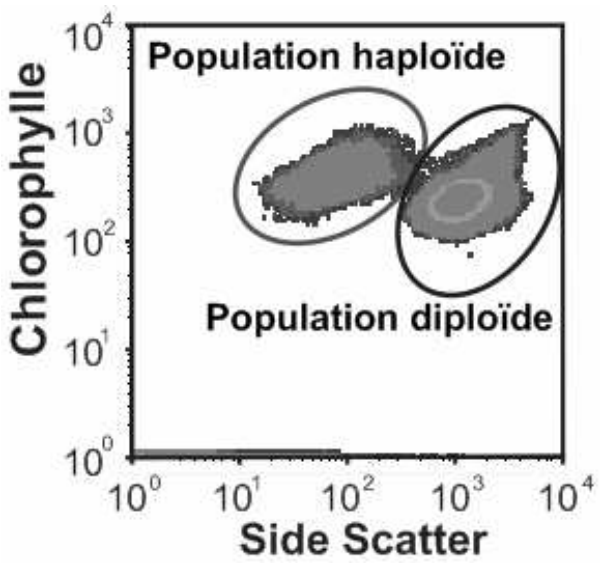


Figure 7 : Profile de cytométrie en flux des deux phases du cycle de vie d'*E.huxleyi*. La phase haploïde (N) possède un 'side scatter' plus bas par rapport à la phase diploïde due à l'absence ou à la présence de coccolithes. Le contenue en chlorophylle des deux stades est le même.

II.4.2. PAM

Les mesures du PAM (Pulse Amplitude Modulated) ont été réalisées à l'aide d'un phyto-pam (phytoplankton analyzer, Walz-Mess und Regeltechnik) et consistaient en la mesure des taux minimum et maximum d'émission de fluorescence après 15 minutes d'adaptation des échantillons à l'obscurité (*Fo* et *Fm* respectivement). Avec les différentes séries de mesure de *Fo* et *Fm, Fv*/*Fm* a pu être calculée (avec *Fv* étant la part variable de cette fluorescence calculé comme : *Fv = Fm – Fo)*. Ce paramètre est une mesure de l'efficacité intrinsèque du photosystème 2 (PS II) qui est considéré comme étant la partie la plus vulnérable de l'appareil photosynthétique en cas de dommages induits par la lumière (voir figure 8).

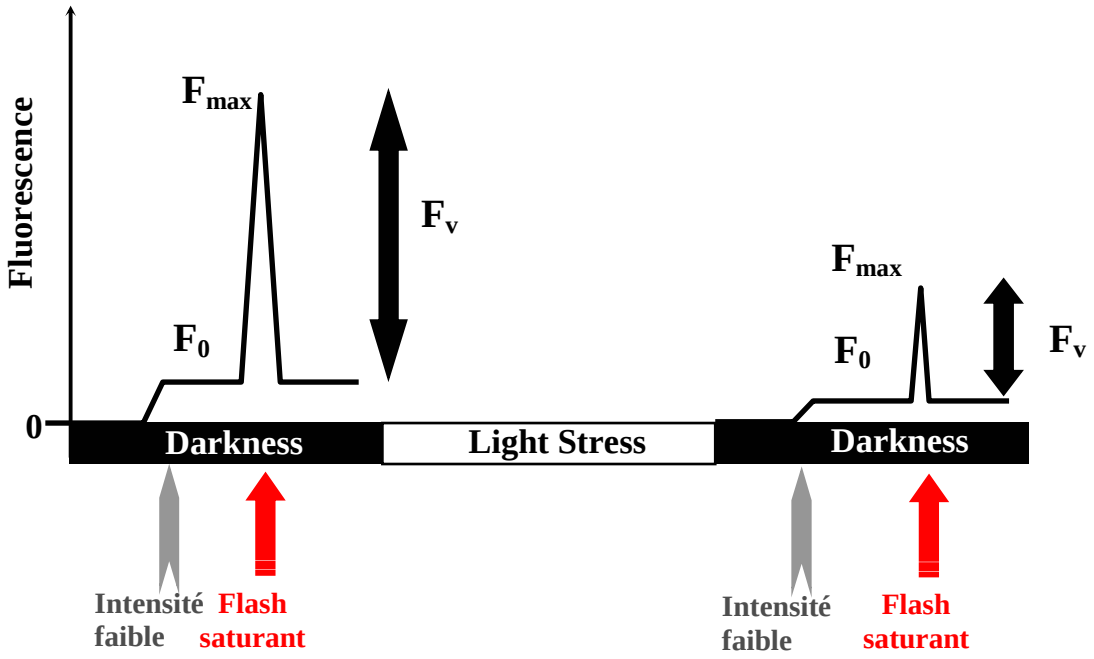


Figure 8 : Schéma de la méthode PAM utilisée pour la mesure de la capacité photosynthétique des cellules. Le paramètre utilisé lors des analyses corresponde à Fv/Fm ((Fmax-F0)/Fmax) qui reflète l'efficacité maximum du photosysthème II. Une diminution de Fv/Fm indique un dommage du centre réactionnel du PSII (photoinhibition).

II.4.3. Dosage sels nutritifs

Les méthodes analytiques de référence pour les nitrites et nitrates sont respectivement la méthode de (Benschneider et Robinson, 1952) et de (Wood et al., 1967), telle que présentée dans son principe par (Strickmand et Parson, 1972), intégrant de nombreuses modifications, et adaptée aux techniques d'autoanalyse. Les mesures sont effectuées en analyse automatique sur des chaînes d'analyse AAII Technicon selon les procédures décrites dans (Tréger et LeCorre,

1975). Dans ces conditions, la précision de mesures est de **± 0.1 µMol/L** au niveau de 10 µMol/L.

Les phosphates sont mesurés selon la méthode décrite par (Murphy et Riley, 1962). Le protocole est celui décrit dans (Aminot et Kérouel, 2004). Les mesures sont effectuées en analyse automatique sur des chaînes d'analyse AAII Technicon selon les procédures que pour les nitrates. Dans ces conditions, la précision de mesures est de **± 0.01 µMol/L** au niveau de 0.40 µMol/L.

L'azote ammoniacal (NH_4) est mesuré selon la méthode de (Koroleff, 1969). Les protocoles de prélèvement, de mesure et d'étalonnage de référence sont ceux exposés en détail dans (Aminot et Kérouel, 2004). La gamme d'étalonnage est réalisée à partir d'eau de mer pauvre en ammonium (eau de mer filtrée sur une soie de 60 µm, vieillie). Le blanc-réactif est réalisé avec de l'eau Milli-Q.

II.4.4. CARD-FISH

A la suite de la préparation des filtres lors de l'expérience de broutage la méthodologie a été la suivante :

1) Perméabilisassions des parois des bactéries avec lysozyme : Les filtres sont découpés en morceau et incubés pendant 60 minutes à 37ºC avec une solution de lysozyme (1mL EDTA 0,5M ; 1mL Tris-HCL 1M pH8 ; 8mL H_2O MilliQ ; 100mg de lysozyme).
2) Lavage I. a)Les filtres ont été lavés en 50mL d'eau MilliQ et puis avec de l'éthanol 100%. b) Les filtres ont été séchés a température ambiante.
3) Hybridation : Les filtres ont été incubés avec du tampon d'hybridation (50mL de tampon composé de : 3,6mL NaCl 5M, 0,4mL Tris-HCl 1M, 50 µl SDS 10%, 3mL H_2O, 11mL de formamide, 2mL d'agent bloquant) et une sonde oligonucleotidique bactérienne (Eub 338 : 5'-CGTGCCTCCCGTAGGAGT-3') à une concentration de 50 ng μl^{-1}. L'hybridation a été mené pendant 2 heures à 35ºC.
4) Lavage II : Les filtres ont été lavés avec 50mL de tampon de lavage II pendant 5min a 37ºC (pour 50mL : 0,5mL EDTA, 1 mL Tris-HCl 1M, 30 µl de NaCl, 50µl de SDS 10%, de l'eau MilliQ a été ajoutée jusqu'à 50mL).
5) Réaction enzymatique avec l'enzyme horseradis peroxidase (HRP) (pour amplifier le signal fluorescent des sondes). Les filtres ont été placés dans un tube eppendorf et incubés pendant 15min à 46ºC avec une mélange de 1mL tampon d'amplification (2mL de tampon phosphate (PBS) 2x concentré, 0,4mL d'agent

bloquant, 16mL de NaCl 1M, 40mL d'eau MilliQ, 4g de dextran sulfate), 10µl de H_2O_2 et 4µl de fluorescéin-tyramide (le fluorochrome).

6) Lavage III : Les filtres ont été lavés avec de l'eau MilliQ et après avec de l'éthanol 100%
7) Coloration des acides nucléiques avec une solution de DAPI et citifluor.
8) Montage des filtres sur une lame en verre pour observation microscopique (microscope- Axio Observer. Zeiss1 ; objectives plan.apochromat 63x/1.40 oil DIC M27 ; fluorescent vert- sonde-HRP et UV – DAPI. Photographies prises avec une AxiocamMR3).

II.4.5. Analyses des données

Pour toutes les expériences où on a fait un suivi de concentration cellulaire, les taux de croissance ont été calculés comme la pente du log naturel du nombre de cellules versus le temps pendant la phase exponentielle de croissance. Pour l'expérience de compétition, un MLG (Modèle Linéaire Générale) a été fait pour tester des interactions entre le milieu (K/2 et K/10), le traitement (proportions relatives entre les deux phases du cycle de vie d'*E.huxelyi*) et la ploïdie (N et 2N) sur le taux de croissance. Pour les autres expériences, les comparaisons des taux de croissance ont été faites par des tests no paramétriques (Mann-Whitney- Kruskal-wallis).

Pour l'expérience de compétition on a voulu aussi explorer les réponses des deux phases à travers l'application d'un modèle classique de compétition: le modèle de Lotka-Volterra. En appliquant ce modèle on considère les stades N et 2N comme des espèces différentes pour lesquelles la croissance est limitée par la capacité limite du milieu et la compétition interspécifique (« inter-cycle ») (Fig. 9A) ((Volterra, 1926)}; (Lotka, 1934)). Cette assomption nous permet de considérer les individus d'un des phases comme des individus de l'autre phase et pouvoir faire des comparaisons pertinentes.

Equation de Lotka-Volterra : $$\frac{dN_1}{dt} = r_1 N_1 \left(\frac{k_1 - N_1 - \alpha N_2}{k_1} \right)$$

Ce modèle simule la situation où deux espèces sont en compétition dans un même environnement. L'impact de l'espèce 2 sur l'espèce 1 est illustré par le coefficient de compétition α, qui représente la place prise par un individu de l'espèce 2 aux dépens de ceux de l'espèce 1. L'impact de l'espèce 1 sur l'espèce 2 est pour sa part illustré par le coefficient

β, qui représente la place prise par un individu de l'espèce 1 aux dépens de ceux de l'espèce 2; α et β sont donc une mesure de l'impact des individus d'une population sur la croissance de la population de l'espèce compétitrice. Ainsi, si α et β = 1, les individus des deux espèces ont un impact équivalent sur la population de leur compétiteur. Par contre, si b = 4, chaque nouvel individu de l'espèce 1 diminuera la croissance de la population de l'espèce 2 de façon équivalente à l'ajout de 4 individus de l'espèce 2 (Fig. 9B).

On peut alors obtenir les valeurs de α et β dans l'équilibre pour chaque traitement et chaque milieu de culture (les valeurs de α et β obtenus pour toutes les situations de compétions est résumé dans le tableau 3)

Selon le modèle, la compétition interspécifique peut donner quatre résultats possibles:

1) $k2\alpha/k1<1$ et $k1\beta/k2>1$ → L'espèce 1 élimine à l'espèce 2
2) $k2\alpha/k1>1$ et $k1\beta/k2<1$ → L'espèce 2 élimine à l'espèce 1
3) $k2\alpha/k1<1$ et $k1\beta<1$ → Les deux espèces coexistent dans un équilibre stable
4) $k2\alpha/k1>1$ et $k1\beta/k2>1$ → Les deux espèces coexistent dans un équilibre instable

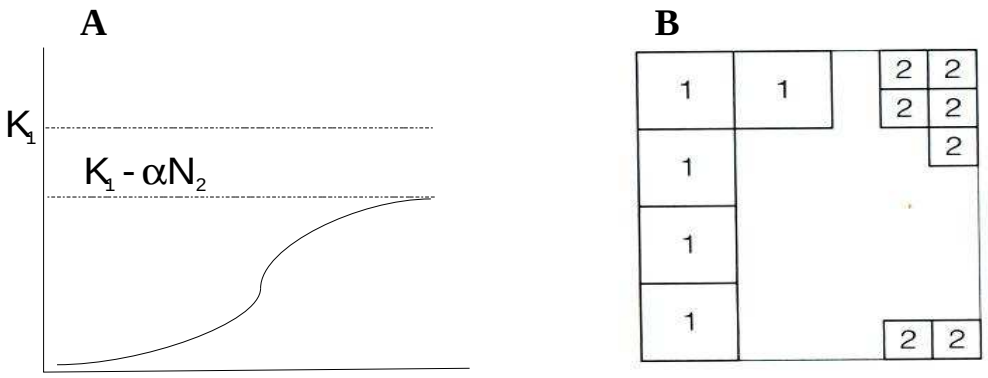


Figure 9: A) **Modèle de Lotka-Volterra** : Croissance limitée par la capacité limite du milieu et la compétition interspécifique: B) Exemple d'impact d'une espèce (phase du cycle) sur l'autre dans le modèle de Lotka-Volterra. Dans cet exemple, chaque nouvel individu de l'espèce 1 diminuera la croissance de la population de l'espèce 2 de façon équivalente à l'ajout de 4 individus de l'espèce 2.

III Résultats

III.1. Compétition entre les stades du cycle de vie

Diverses proportions de cellules N et 2N d'*E. huxleyi* ont été cultivées en milieu de culture K/2 et K/10 et les densités cellulaires des cultures ont été suivies par cytométrie en flux.

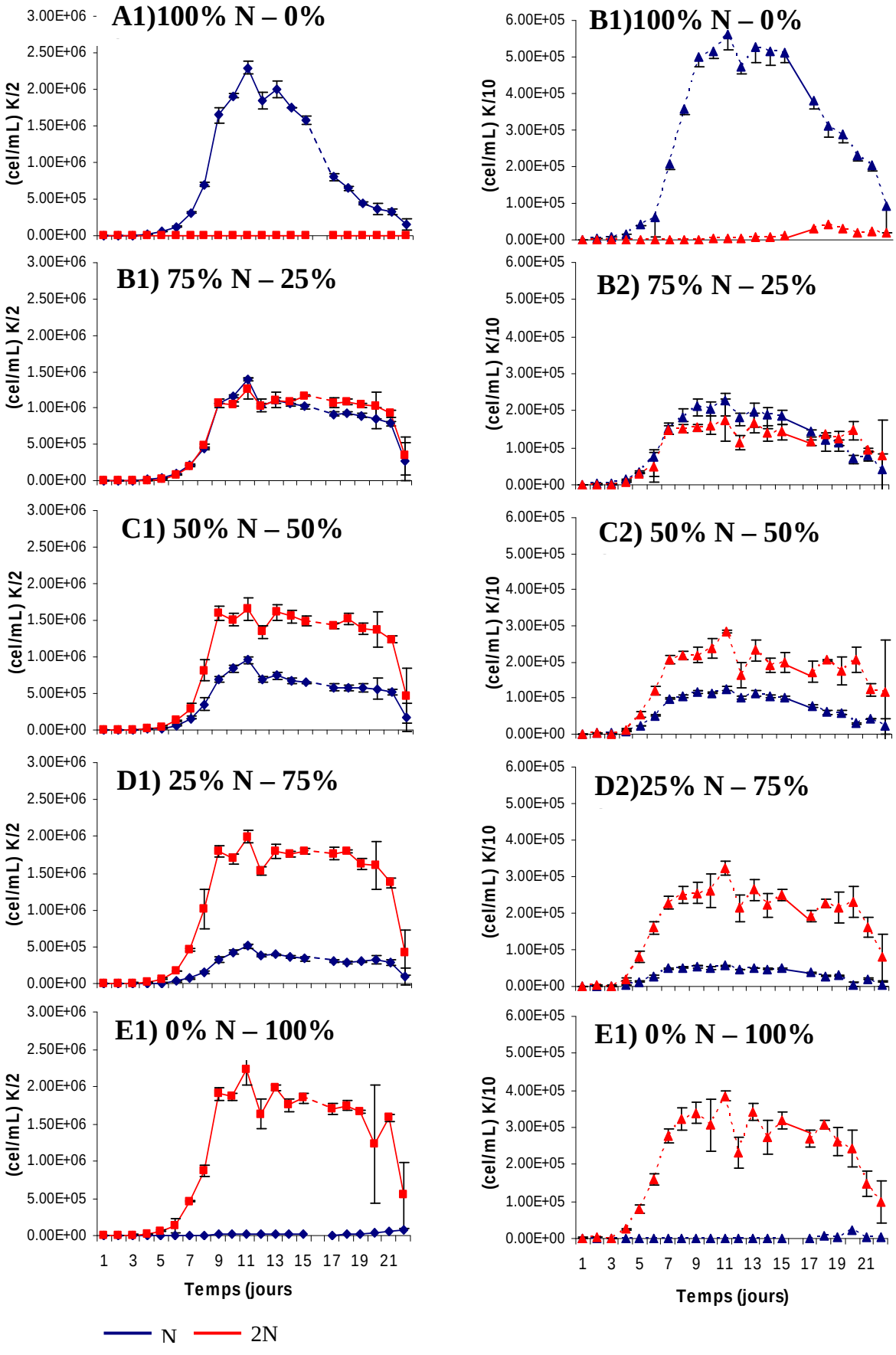


Figure 10: Courbes de croissance des expériences de compétition entre les phases N et 2N d'*E.huxleyi* dans milieu K/2 (à gache) et dans le milieu K/10 (à droite).
A) 100%N - 0%2N, **B)** 75%N - 25%2N, **C)** 50%N - 50% 2N, **D)** 25%N - 75%2N, **E)** 0%N - 100%2N. Les lignes bleues représentent les cellules N et les lignes rouges les cellules 2N.

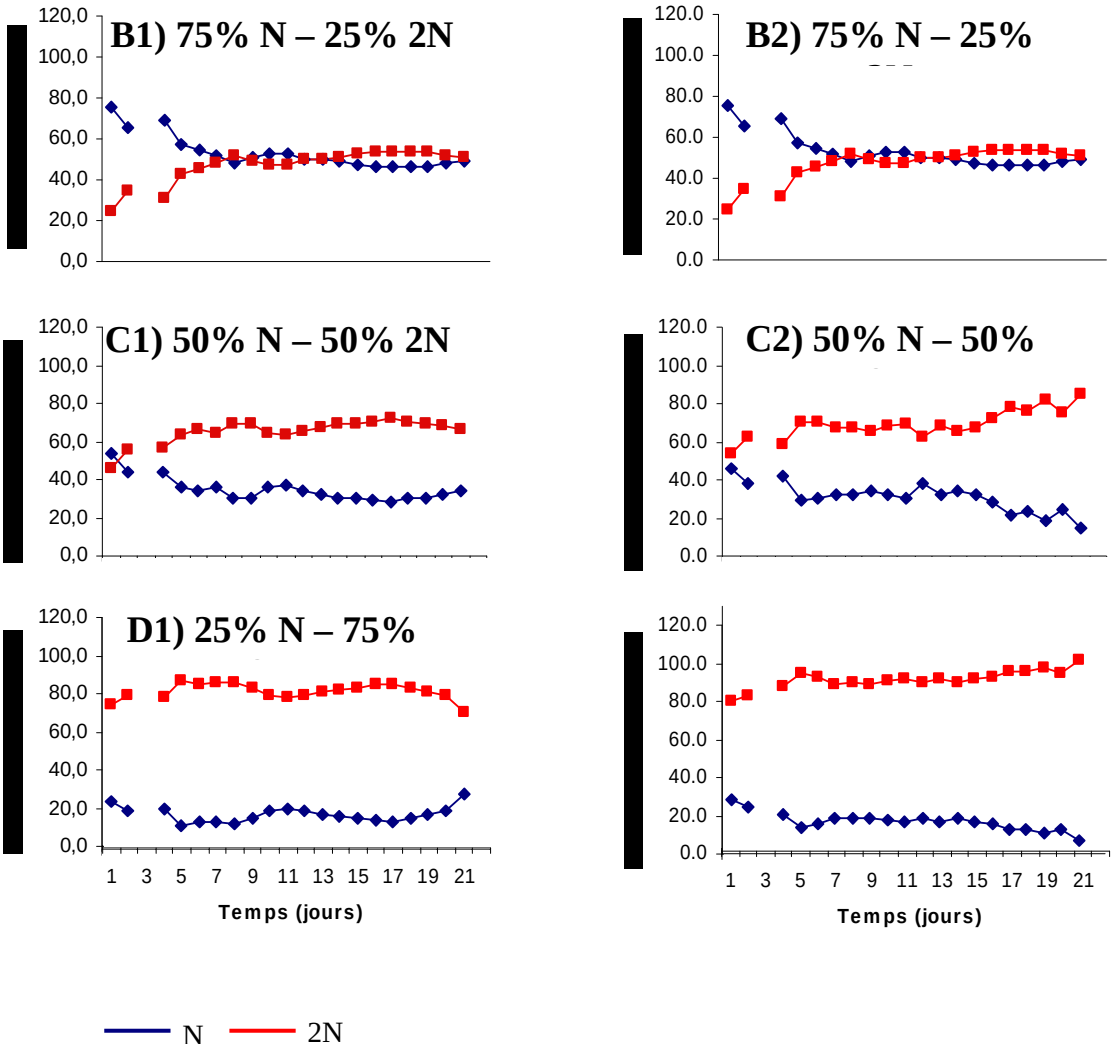


Figure 11: compétition entre les phases N et 2N d'*E.huxleyi* dans le milieu K/2
Les graphes montrent les proportions relatives (pourcentages) des deux phases du cycle par rapport au nombre total de cellules. Les graphes de gauche correspondent aux cultures faites en Milieu K/2 et graphes de droite aux cultures faites en milieu K/10. **A)** 100%N - 0%2N, **B)** 75%N – 25%2N, **C)** 50%N - 50%2N **D)** 75%N - 25%2N, **E)** 100%N - 100% 2N. Les pourcentages de cellules haploïdes sont représenté en bleue et ceux de cellules diploïdes en rouge.

Les cultures de contrôles (100% de cellules N ou 2N) ont été maintenues monophasiques durant toute l'expérience (Fig. 10, A et E). Dans toutes les cultures, monophasiques ou mixtes, les concentrations cellulaires en phase stationnaire sont supérieurs en milieu riche K/2 (de l'ordre de 10^6 cellules par ml) par rapport au milieu comparativement pauvre K/10 (de l'ordre de 10^5 cellules par ml). De plus, la phase exponentielle de croissance est plus longue en milieu riche (9 jours) qu'en milieu limité (5 jours). Ces variations reflètent les différents taux de croissance dans ces deux milieux.

Dans les cultures mixtes, les taux de croissance des cellules haploïdes d'une part et diploïdes d'autre part sont similaires dans les trois conditions de mélanges (B, C et D). En revanche, celui des cellules diploïdes est toujours supérieur à celui des cellules haploïdes. Ainsi, le pourcentage de cellules diploïdes lorsque les cultures atteignent leur phase stationnaire est supérieur au pourcentage du mélange initial (Fig. 11). Cette tendance est d'autant plus visible que la proportion de cellules de départ est favorables aux haploïdes. Par exemple, le pourcentage de cellules diploïdes en B1 est de 25 % dans le mélange initial et il passe à environ 50 % en phase stationnaire (soit une augmentation de 100 %). En revanche lorsque le pourcentage de cellules diploïdes dans le mélange initial est de 75 %, il est de 80 % environ en phase stationnaire (soit une augmentation de 7 % environ). D'une manière générale, dans les conditions expérimentales utilisées, les cellules diploïdes croissent plus facilement que les cellules haploïdes.

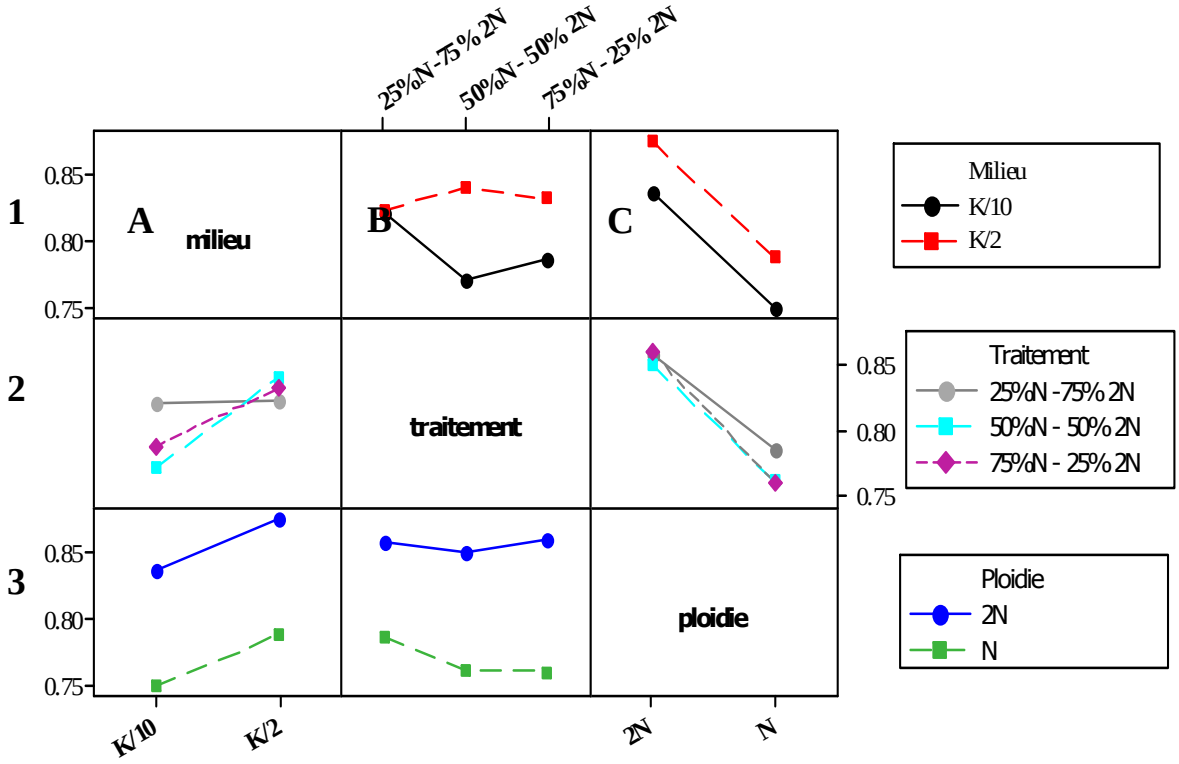


Figure 12: modèle linéaire général à trois facteurs : Milieu (K/2, K/10), Traitement (75%N-25%2N, 50%N-50% 2N, 25%N-75%2N) et Ploïdie (N, 2N). Les graphiques dans la figure montrent les interactions entre ploïdie, traitement et milieu pris deux à deux. Les axes latérales (0.75-0.85) son proportionnels au taux de croissance.
Le tableau en bas montre les valeurs p de signification du test. Une valeur p inférieure à 0.05 indique que le facteur correspondant a un effet significatif sur le taux de croissance.

Facteur	P
milieu	**0.011**
traitement	0.645
ploidie	**0.000**
traitement*ploidie	0.746
milieu*ploidie	0.993
milieu*traitement	0.180

Afin de représenter l'influence du milieu, du traitement (proportions des cellules haploïdes et diploïdes de départ), et de la ploïdie, un MLG a été établi à partir des données des taux de croissance (Fig 12). Le haut de la figure représente un tableau croisé entre les trois facteurs. Par exemple en **C1**, quelque soit la ploïdie, le taux de croissance est plus grand en milieu K/2 (en rouge) qu'en milieu K/10 (en noir). En **A3**, quelque soit le milieu, le taux de croissance des cellules diploïdes (en bleu) est supérieur à celui des cellules haploïdes (en vert). Enfin, en **B3**, quelque soit le traitement, le taux de croissance des cellules diploïdes (en bleu) est supérieur à celui des cellules haploïdes (en vert). Le tableau en bas de figure représente les résultats numériques du modèle où une valeur p inférieur à 0.05 indique que le facteur étudié a un effet significatif sur le taux de croissance. Ces résultats montrent que le milieu de culture et la ploïdies ont un effet notable sur la croissance des cellules. Ces données vérifient que les cellules diploïdes se multiplient mieux que les cellules haploïdes.

Afin d'étudier l'effet d'un type cellulaire sur la croissance de l'autre, un modèle de Lotka-Volterra a été établi pour les deux milieux utilisés. Les valeurs de k2α/k1 et k1β/k2 sont inférieures à 1 quelque soit le traitement appliqué en milieu K/2 (Tableau 3). Ceci signifie que les deux "espèces" (ici, les cellules haploïdes et diploïdes) coexistent dans un équilibre stable, c'est-à-dire que les deux populations sont peu compétitives et qu'aucune des deux n'est capable d'éliminer l'autre. Par contre, les valeurs de k2α/k1 et k1β/k2 en milieu K/10 sont supérieure à 1 quelque soit le traitement appliqué : les deux "espèces" coexistent dans un équilibre instable. Chaque "espèces" abondante inhibe la croissance de l'autre de façon plus importante que sa propre croissance. Les deux phases restent dans un équilibre instable jusqu'à ce qu'une des deux gaigne, dans cette étude sont les cellules diploïdes qui, prennent le dessus.

Tableau 3 : modèle de Lotka-Volterra
Valeurs obtenues pour chaque traitement et chaque milieu de culture. Les valeurs de K_1, K_2, N_1 et N_2 sont en millions.

		Milieu K/2	Milieu K/10
K_1		1.86	0.52
K_2		1.76	0.31
75% N-25% 2N	N_1	0.92	0.19
	N_2	0.12	0.15
	α	0.89	2.2
	β	0.76	0.84
	k2α/k1	**0.84**	**1.30**
	k1β/k2	**0.80**	**1.40**
50% N-50% 2N	N_1	0.58	0.11
	N_2	1.45	0.2
	α	0.88	2.08
	β	0.53	1.06
	k2α/k1	**0.83**	**1.24**
	k1β/k2	**0.56**	**1.76**
25% N-75% 2N	N_1	0.3	0.048
	N_2	1.78	0.25
	α	0.88	1.92
	β	-0.06	1.36
	k2α/k1	**0.83**	**1.15**
	k1β/k2	**-0.06**	**2.26**

III.2. Influences des phases du cycle d'*E. huxleyi* sur le milieu

III.2.1. Effets sur le pH

Afin de savoir si les cellules haploïdes et diploïdes influent de manière différente sur le milieu, les valeurs de pH des cultures ont été mesurées durant l'expérience de compétition (Fig. 13). En milieu K/2, les valeurs de pH augmentent par rapport à la valeur de pH initiale (pH = 8.0). Cette augmentation de pH semble proportionelle avec le pourcentage initial de cellules haploïdes dans la culture. Les cultures monophasiques confirment qu'une culture de cellules haploïdes rend le milieu plus basique qu'une culture de cellules diploïdes.

En milieu K/10 l'augmentation de pH suit également le pourcentage initial de cellules haploïdes. Cependant, les cellules diploïdes modifient peu le pH. L'effet des cellules sur le pH est donc dépendant du milieu.

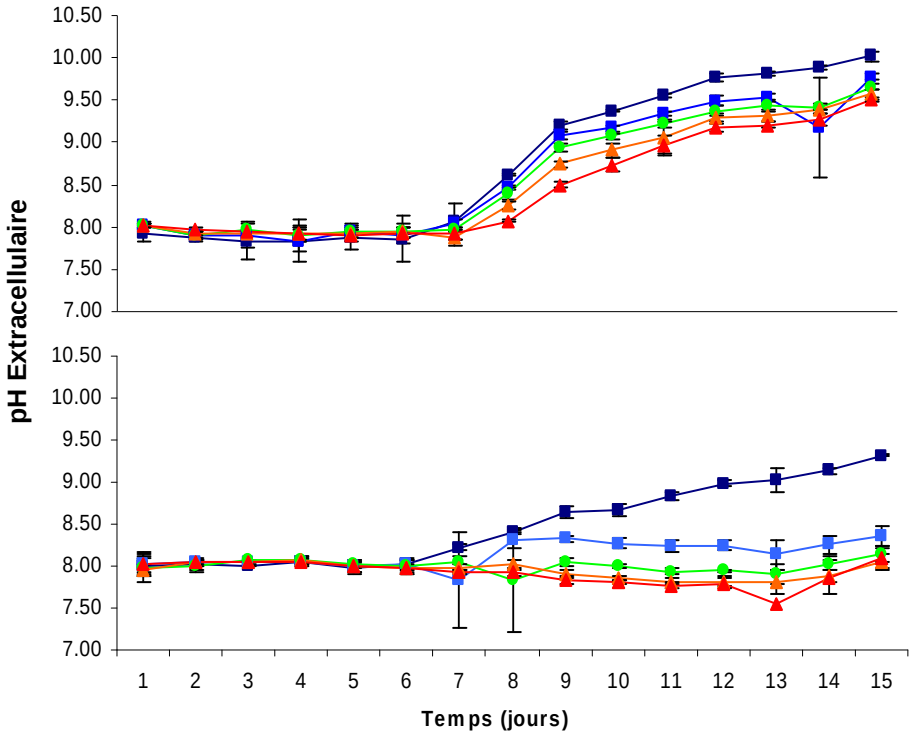


Figure 13 : Suivie du pH des cultures lors de l'expérience de compétition entre les stades N et 2N *d'Emiliania huxleyi*. Le graphique A montre les résultats en milieu K/2 et le graphique B en milieu K/10.

III.2.2. Effets sur les sels nutritifs

Afin de comparer la consommation des sels nutritifs pas les cellules haploïdes et diploïdes, des mesures de concentrations de nitrates, de nitrites, de phosphates et d'ammonium ont été réalisées durant la phase exponentielle de cultures monophasiques en milieu K/10 (Fig. 14). Les concentrations basales de nutriments mesurées dans le milieu seul (c'est-à-dire en l'absence de cellules) ont été de 110 µM pour les nitrates, 0.05 µM pour les nitrites et 0.22 µM pour les phosphates.

La diminution de la concentration des phosphates est importante et sont épuisés au bout de 4 jours dans les deux cultures (Fig. 14B). Les phosphates deviennent donc un facteur limitant. La consommation de phosphates par cellule est proportionnelle à la concentration présente dans le milieu.

L'évolution de la concentration de nitrates dans le milieu (Fig. 14C, ligne continue) diminue avec le temps et cette diminution est significativement plus importante dans les cultures 2N que dans les cultures N (Tableau 2). La consommation de nitrates par les cellule (ligne pointillée) est plus intense au 3ème jour dans les deux cultures. Finalement, la

concentration des nitrites, augmente dans le milieu de culture des cellules diploides. Ainsi, celles-ci consomment plus rapidement les nitrates et produisent plus de nitrites que les cellules haploïdes.

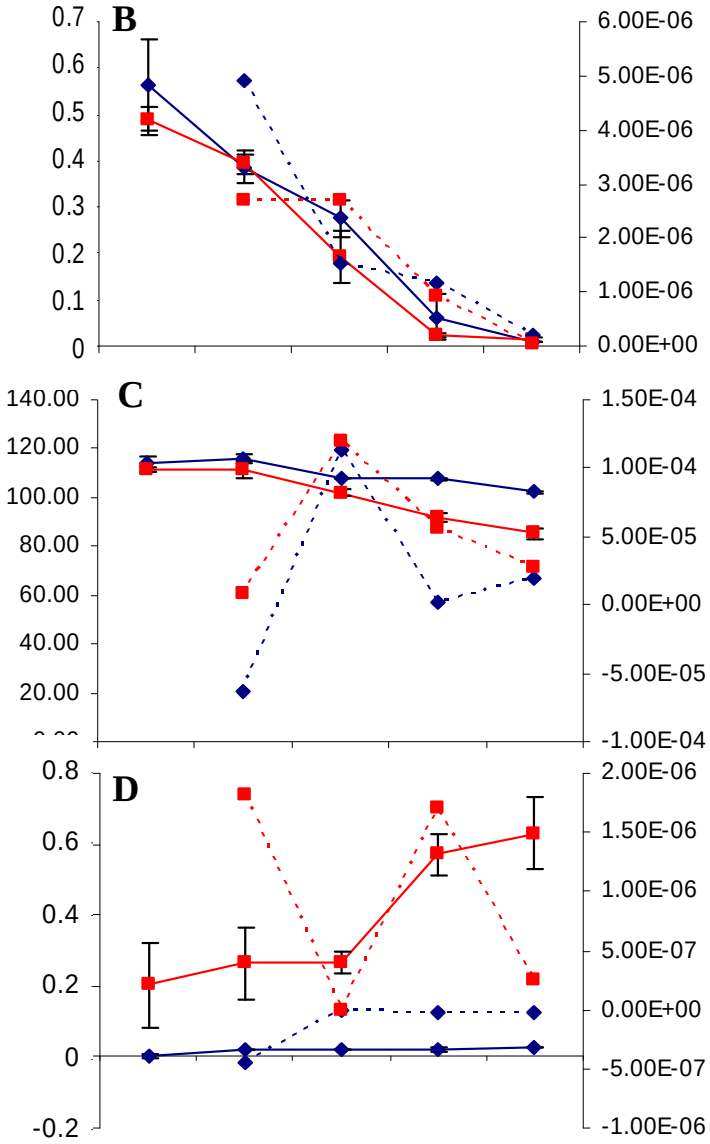


Figure 14 : suivi des sels nutritifs pour les stades N et 2N d'*E. huxleyi* en milieu K/10. Ces graphes montrent : A) Courbe de croissance cellulaire en échelle logarithmique, B→E) Evolution des concentrations extracellulaires de nutriments (axe gauche, traits continus) et consommation des nutriments par jour par cellules (axe droite, trait pointillé). B) Phosphates, C) Nitrates, D) Nitrites. Les lignes bleues représentent les cultures haploïdes et les lignes rouges les cultures diploïdes.

Tableau 4 : Taux de croissance cellulaire et taux de consommation des nutriments pour les cultures N et 2N. Les valeurs représentent les moyennes ± ecartype. Les étoiles indiquent les situations où il y a des différences significatives entre les réponses de N et 2N (Mann-Whitney, $p<0.05$).

	Taux croiss.	NO3 *	NO2 *	PO4	NH4
N	0,77 ± 0,06	3,17 ± 0,75	0,01 ± 0,001	0,16 ± 0,02	0 ,08 ± 0,10
2N	0,97 ± 0,18	7,18 ± 0,18	0,12 ± 0,01	0,16 ± 0,01	0, 01 ± 0,02

III.3. Evaluation des facteurs de répression/inhibition potentiels entre les phases du cycle de vie.

Les expériences précédentes ont montré que les cellules haploïdes modifient de manière plus importante le pH du milieu que les cellules diploïdes, et que les cellules diploïdes consomment plus rapidement les nitrates. Les deux phases cellulaires ont donc des effets différents sur le milieu de culture. Afin de savoir si la compétion entre les deux phases cellulaires se fait *via* le milieu (par exemple à travers le pH, les nutriments consommés ou excrétés, ou d'autres facteurs sécrétés), des cultures de cellules haploïdes ont été réalisées en milieu issu de cultures diploïdes et inversement (Fig. 15). Les taux de croissance sur les deux milieux ont été comparés et aucune différence significative n'a été détectée, que ce soit pour les cultures de cellules haploïdes ou diploïdes.

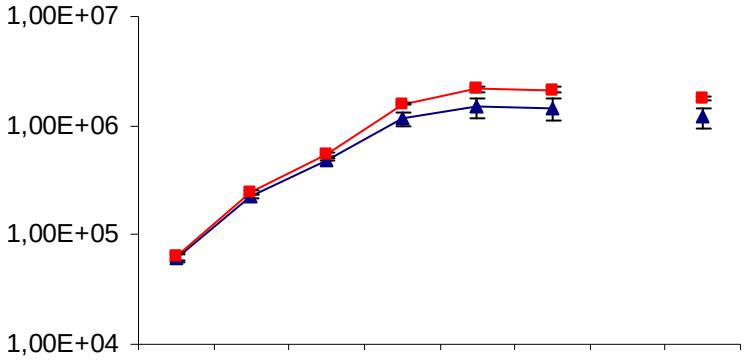


Figure 15 : évaluation des facteurs de répression/inhibition potentiels entre les stades N et 2N *d'Emiliania huxleyi.*
Ces graphes montrent les courbes de croissance cellulaire (en échelle logarithmique) obtenues pour les cultures haploïdes (A) et pour les cultures diploïdes (B). Les lignes bleues représentent les cultures faites en « milieu N » et les lignes rouges les cultures faites en « milieu 2N ». Les lignes pointillées indiquent des échantillons manquants.

III.4. Influence de l'intensité lumineuse

Des cultures monophasiques haploïdes et diploïdes ont été réalisées en milieu K/10 et les taux de croissance et l'activité photosynthétique ont été comparés sous différentes intensités lumineuses.

III.4.1. Croissance des cultures

Les taux de croissance des cellules haploïdes et diploïdes présentent une valeur maximale sous une intensité lumineuse moyenne (Fig. 16), suggérant la proximité d'une intensité lumineuse optimale pour la croissance. A basse lumière, les taux de croissance des deux cultures sont similaires et très diminués. .En revanche, dans les conditions d'intensité lumineuses les plus fortes, la croissance de la phase haploïde est nettement réduite alors que celle de la phase diploïde n'est que peu modifiée par rapport à la croissance à moyenne lumière.

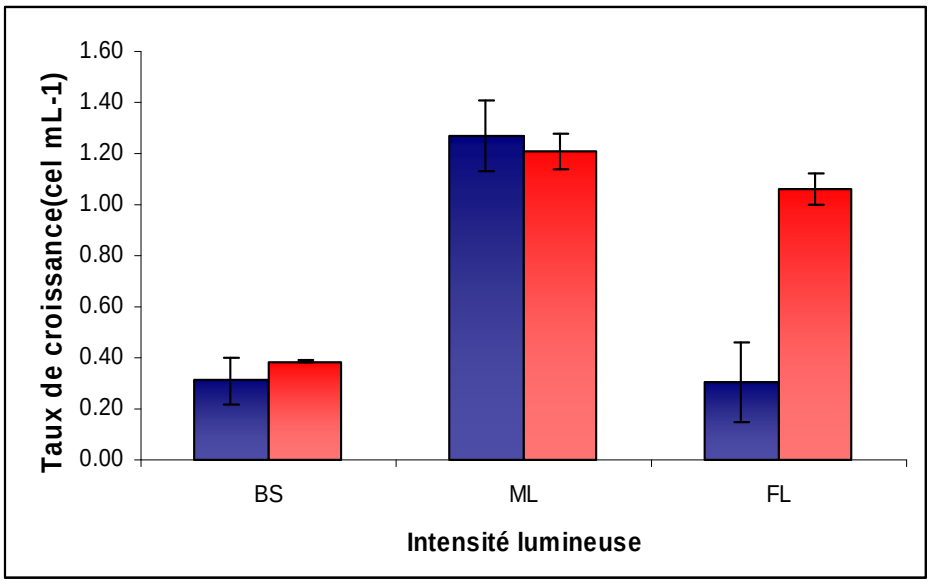


Figure 16: réponse des deux phases du cycle de vie d'*E.huxleyi* à des différentes intensités lumineuses
Taux de croissance des cultures N (en bleu) et 2N (en rouge) en basse lumière (BL : ~ 15 µE.m^{-2}.s^{-1}), moyenne lumière (ML : ~ 100 µE.m^{-2}.s^{-1}), et forte lumière (FL : ~ 300 µE.m^{-2}.s^{-1}). Les différences significatives entre les taux de croissance des deux phases sont indiqués par une étoile (Kruskal-Wallis, $p<0.05$).

III.4.2. Activité photosynthétique

Les valeurs de Fv/Fm, qui sont une mesure de l'activité photosynthétique, ont été obtenues pendant la phase exponentielle de croissance (Fig. 17). Sous faible et moyenne intensité lumineuse le rendement quantique optimal (Fv/Fm) ne diffère pas significativement entre les deux phases du cycle. L'augmentation de l'intensité (FL) s'accompagne d'une diminution du rendement quantique pour les cellules de la phase haploïde qui n'a pas lieu dans la phase diploïde. Ainsi, à de fortes intensités lumineuses, les cellules diploïdes présentent une activité photosynthétique et un taux de croissance plus importants.

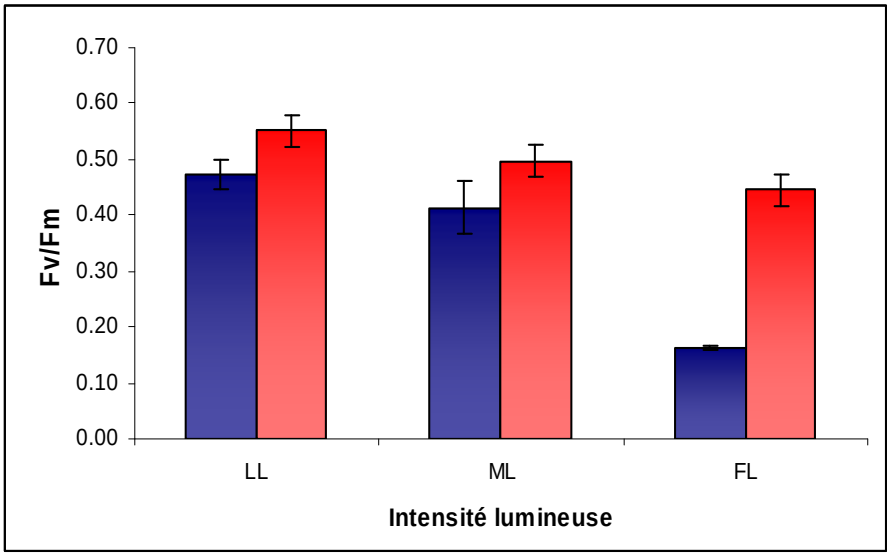


Figure 17 : activité photosynthétique des deux phases du cycle de vie d'*E.huxleyi* à des différentes intensités lumineuses
Les valeurs moyennes de Fv/Fm lors de la phase exponentielle de croissance pour les cultures N (en bleu) et 2N (en rouge) ont été mesurés aux différentes intensités lumineuses déjà décrites (cf. Fig. 14). Les différences significatives entre les Fv/Fm des deux phases phases sont indiquées par une étoile (Kruskal-Wallis, $p<0.05$).

III.4. Expérience 4 : Broutage

L'utilisation de la technique de CARD-FISH a été utilisée pour mettre en évidence l'existence de bactéries à l'extérieur et/ou à l'intérieur de plusieurs espèces d'haptophytes (Fig. 18). Malgré une relativement forte autofluorescence parasite verte provenant des chloroplastes des haptophytes, le CARD-FISH a permis la détection spécifique de bactéries ainsi que leur localisation intra/extracellulaire.

La présence de bactéries (marquées en vert par la fluorescéine) a été détectée dans le cytoplasme de la phase haploïde de deux espèces de coccolithophore, *E.huxleyi* (A) et *Jomonlithus* sp. (D), ainsi que dans celui d'une autre espèce d'haptophyte (non-coccolithophore) de ploïdie inconnue, *C. camella* (H). En revanche, dans les autres espèces étudiées, des bactéries ont été détectées seulement à l'extérieur des cellules (B, C, E, F, G, I).

Ainsi, des bactéries intracellulaires ont été détectées dans trois espèces d'haptophytes, dont deux coccolithophores. Chez ces derniers et dans les conditions expérimentales proposées, seules les phases haploïdes sont mixotrophes, et donc capables d'acquérir des nutriments à la fois par voie photosynthétiques et par phagocytose.

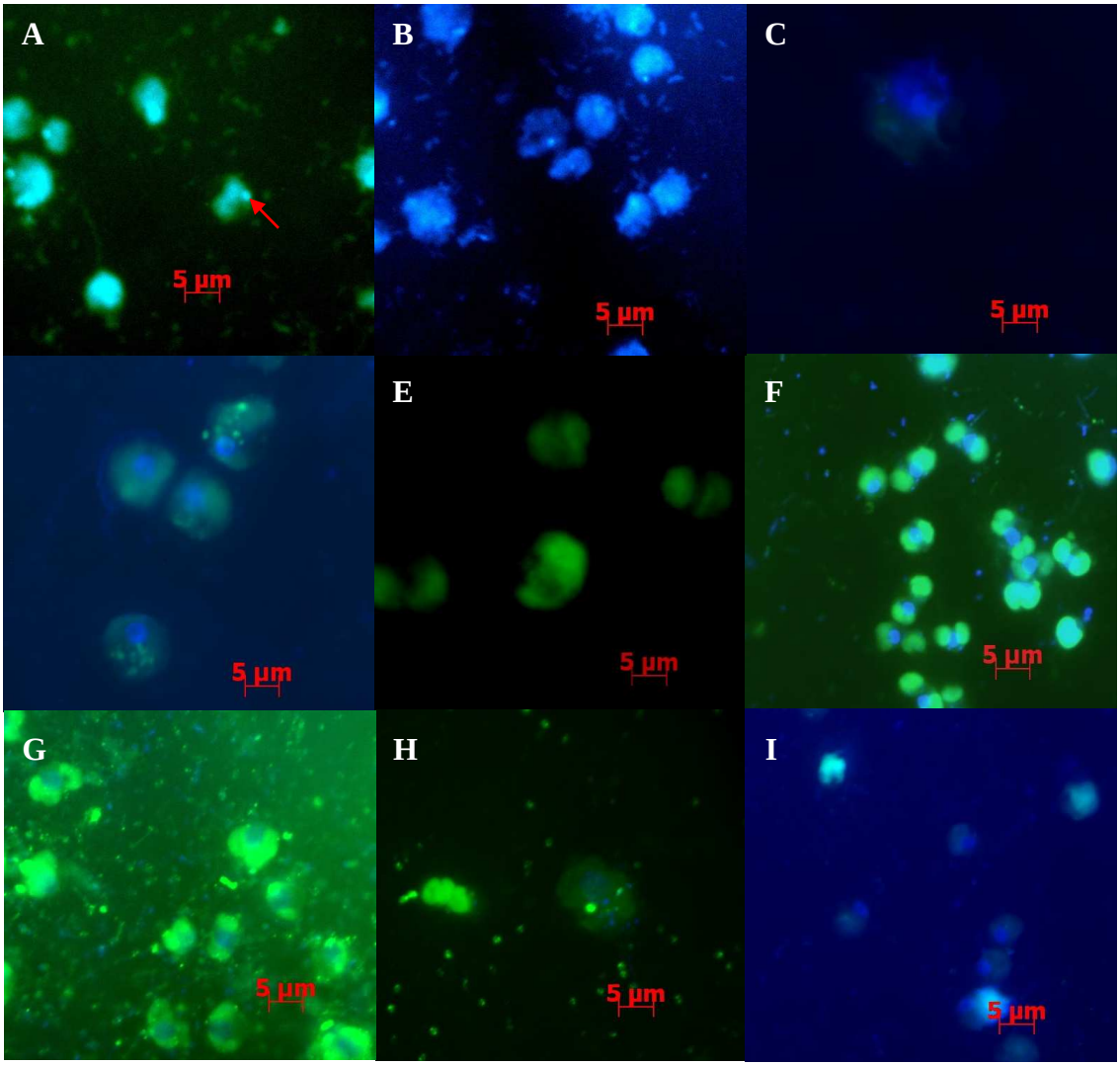


Figure 18: études de mixotrophie des algues haptophytes.
Les bactéries co-habitantes des haptophytes sont marquées en vert par une sonde moléculaire fluorescente (par la technique d'hybridation *in situ– FISH*). Les acides nucléiques sont marqués en bleu par le DAPI. Des bactéries sont détectées à l'extérieur des haptophytes et dans certains cas à l'intérieur des haptophytes (indiqué par les flèches rouges). Les photos on été prises à une amplification de 63x.

A. *Emiliania huxleyi,* AC472 (Haploïde) – **mixotrophie**
B. *Emiliania huxleyi,* AC472 (Diploïde) - inexistence de mixotrophie
C. *Schyphosphaera apstenii,* AC504 (Ploïdie inconnue) - inexistence de mixotrophie
D. *Jomonlithus* sp., AC513 (Haploïde) - **mixotrophie**
E. *Jomonlithus* sp., AC513 (Diploïde) - inexistence de mixotrophie
F. *Prymnesium parvum* ,AC34 (Ploïdie inconnue) - inexistence de mixotrophie
G. *Calyptrosphaera sphaeroidea,* AC507 (Ploïdie inconnue) - inexistence de mixotrophie
H. *Crysochromulina camelle,* AC56 (Ploïdie inconnue) - **mixotrophie**
I. *Pleurochrysis carterae,* AC1 (Diploïde) - inexistence de mixotrophie

IV. Discussion

Cette étude apporte une série de résultats fondamentaux visant à comprendre la physiologie des stades de vie haploïde et diploïde chez les coccolithophores, leur interaction et leur influence sur l'environnement. Les différentes expériences d'écophysiologie présentées dans ce travail portent sur deux facteurs environnementaux majeurs en milieu marin, les nutriments et la lumière, ainsi que sur un métabolisme qui pourrait être très répandu dans l'océan oligotrophe : la mixotrophie.

Les résultats de l'expérience de compétition montrent que la phase calcifiante diploïde *d'E. huxleyi* est plus performante que la phase mobile haploïde sous les conditions expérimentales utilisées**.** La comparaison des taux de croissances montre que la phase diploïde présente des taux plus élevés dans les deux milieux de cultures utilisés et pour tous les traitements (Fig.12). De plus, selon les prédictions du modèle de Lotka-Volterra, l'avantage de la phase 2N devient encore plus marqué lorsque le milieu de culture est limité en nutriments. Dans ces conditions qui ressemblent davantage au milieu naturel, les cellules subissent un équilibre instable au cours duquel la phase la plus performante (2N) pour l'utilisation des ressources devrait largement surpasser, sinon exclure la phase haploïde.

La phase à coccolithes d'*E. huxleyi* est réputée pour son affinité très importante pour les nutriments, ce qui expliquerait en partie son succès écologique ((Egge et Heimdal, 1994); (Riegman et al., 2000); (Smayda, 1997) notamment dans des milieux plus ou moins carencés. Toutefois la phase haploïde flagellée semble moins compétitive dans ce type de milieu, ce qui s'expliquerait par une affinité moindre pour les nutriments.

Le suivi de sels nutritifs durant la croissance pourrait apporter une explication aux tendances observées dans les expériences de compétitions. On constate en effet que les cellules diploïdes provoquent une diminution des nitrates significativement plus importante que celle produite par les cellules haploïdes (Fig.14C). Cette différence est probablement due aux métabolismes distincts des deux phases. L'intense consommation des nitrates observée en début de phase exponentielle (jour 2, Fig. 12) est un comportement caractéristique, de type "surge–uptake" (littérallement "consommation d'urgence"), et décrit chez d'autres organismes phytoplanctoniques dans des situations de faible concentration en nutriments ((Flynn, 2003) ; (Stolte et al., 1994)). Les cellules absorbent alors les sels nutritifs disponibles à leur capacité maximale et les gardent en réserve. Dans la phase diploïde, la consommation des nitrates s'accompagne d'une production de nitrites (14D). Cette production

pourrait être une conséquence de l'excès de nitrates absorbés qui n'arrivent pas à être assimilés et qui sont donc éliminés sous forme de déchets.

Un facteur qui pourrait expliquer en partie l'excès de nitrates absorbés (et en conséquence l'élimination des nitrites) dans la phase diploïde est le déséquilibre entre les concentrations de nitrates et de phosphates intrinsèques au milieu de culture utilisé ($NO_3/PO_4 \approx 500$). En 1934, Redfield avait observé que, indépendamment des concentrations, il existait un rapport sensiblement constant entre le contenu en nitrate et en phosphate des eaux de mer, et que ces éléments étaient apparemment extraits du milieu ambiant par le plancton dans les mêmes proportions. Cette relation, ou rapport de Redfield, correspond à 16, ce qui est bien inférieur a notre valeur expérimentale.

Cependant, certains auteurs (Riegman et al., 1992) ont postulé qu'un des facteurs environnementaux clés pour le développement des blooms diploïdes d'*E. huxleyi* est justement la limitation en phosphate. Les blooms d'*E. huxleyi* ont lieu seulement lorsque le rapport NO_3/PO_4 est supérieur à 16 et les concentrations cellulaires maximales correspondent à des rapports NO_3/PO_4 supérieurs à 25 {(Tyrrel et Taylor, 1996) ;. (Smayda, 1997) et (Riegman et al., 2000)ont par ailleurs démontré que *E. huxleyi* 2N présente une très forte affinité pour le phosphate dans des situations de stress ($NO_3/PO_4 = 300$), avec une faible constante de demi saturation de 1 nmol L^{-1}. Riegman a aussi montré que le transcriptome d'*E. huxleyi* 2N contient deux enzymes phosphatases alcalines extracellulaires qui permettraient d'utiliser le phosphate organique à de très faibles concentrations, de l'ordre du nanomole. La phase diploïde d'*E. huxleyi* est donc fortement compétitive dans un milieu pauvre en phosphate et avec un rapport NO_3/PO_4 élevé.

Nos conditions expérimentales ne seraient donc pas si éloignées des conditions naturelles. Cependant, le relatif succès de la phase diploïde ne s'expliquerait pas par l'extrême affinité classiquement observée chez *E. huxleyi* pour les phosphates, mais plutôt par un taux de consommation accru des nitrates. Il aurait été intéressant de mesurer l'expression des enzymes phosphatase alcaline et nitrogénase dans les cultures haploïde et diploïde, en milieux riche et pauvre en nutriments. Cependant, une telle expérience nécessite des cultures axéniques car certaines bactéries possèdent les mêmes enzymes. Pendant ce travail, des essaies d'élimination des bactéries contaminantes en utilisant des antibiotiques ont provoqué des malformations (perte de coccolites) dans les cultures diploïdes, ce que a empêché la réalisation de ces expériences. .

Les résultats des expériences de compétitions et de suivi des nutriments sont donc en accord, et suggèrent que le succès relatif de la phase diploïde sur la phase haploïde n'est pas du à une exclusion par info-chimie à travers le milieu. Les expériences de croissance des cultures monophasiques dans des milieux 2N et N confirme cette conclusion (Fig.15).

Si les stades haploïdes et diploïdes n'interfèrent pas l'un sur l'autre à travers l'environnement, ils ont un effet certain sur la chimie environnementale, et en particulier le pH (Fig. 13). La différence observée entre les deux milieux résulte probablement de variations dans l'équilibre entre la photosynthèse et la calcification. On peut supposer que dans un milieu plus riche (K/2), où les concentrations cellulaires sont élevées, la photosynthèse prend une place importante qui contribue à une augmentation de pH (par consommation du CO_2 produit par la respiration). Par contre dans un milieu plus pauvre (K/10), la calcification des cellules 2N pourraient compenser l'action de la photosynthèse.

En plus des différences dans le taux d'assimilation des nutriments, la lumière joue certainement un rôle important dans les différences physiologiques entre les deux phase du cycle de vie chez *E.huxelyi*. Nos résultats montrent clairement que les deux stades de vie ont une préférence pour les intensités lumineuses moyennes, et que les populations haploïdes ne supportent pas les intensités lumineuses fortes (ces valeurs «fortes» restant cependant inférieures aux maxima de lumière en milieu naturel).

Nos données corroborent des travaux antérieurs qui ont montré que les taux de croissance chez *E. huxleyi* 2N augmentent avec le PFD (Photon Flux Density) de 0.38 $jour^{-1}$ à 50 $\mu E.m^{-2}.s^{-1}$ à 0.82 $jour^{-1}$ à 800 $\mu E.m^{-2}.s^{-1}$. Cette corrélation entre taux de croissance et PFD a aussi été reportée par (Nielsen, 1997)et (Harris et al., 2005)qui ont démontré que de fortes PFD n'ont pas d'effet inhibiteur sur le stade calcifiant diploïde d'*E. huxleyi*

Le manque de photoinhibition dans le stade diploïde pourrait lui conférer un avantage compétitif par rapport aux autres organismes phytoplanctoniques, notamment dans des environnements turbulents. En effet, ceci est compatible avec les conditions optimales d'efflorescence de la phase diploïde. Les blooms d'*E. huxleyi* se développent généralement dans des couches mélangées relativement peu profondes (Holligan et al., 1993). La présence de photoinhibition chez la phase haploïde pourrait résulter d'une adaptation à des couches plus profondes dans la colonne d'eau, notamment au niveau du DCM (Deep Chlorophyll Maximum). Une telle séparation spatiale est une hypothèse a tester en milieu naturel à l'aide de la méthode COD-FISH (Frada et al., 2006).

Pour l'état haploïde, l'occurrence de photoinhibition à de fortes lumieres, pourrait indiquer une séparation de niche (spatiale et/ou temporelle) par rapport à la phase diploïde. A ce jour, l'absence de données en milieu naturel concernant la phase haploïde ne fournit aucune indication quant à sa distribution spatiale et temporelle. Cependant cette absence d'informations ne peut être interprétée comme une non-existence de la phase haploïde dans le milieu naturel. Il serait possible que le rôle de cette phase mobile d'*Emiliania huxleyi* soit largement sous-estimé alors qu'elle pourrait avoir une incidence dans l'apparition ou la fin des blooms.

Nos données confirment la possibilité de mixotrophie dans la phase haploïde du cycle de vie des coccolithophores. L'existence de mixotrophie chez les haptophytes est déjà bien connue. Quelques espèces de *Chrysochromulina* et *Prymnesium* peuvent acquérir des nutriments organiques du milieu (bactéries, autres microalgues, autres composants organiques) tout en préservant leurs chloroplastes pour la photosynthèse. Ces espèces sont phylogénétiquement plus primitives, et de Vargas *et al.* (De Vargas et Probert, in press) ont proposé une origine hétérotrophique et côtière des haptophytes, qui seraient devenus au cours de l'évolution des organismes de plus en plus autotrophes et océaniques à partir de l'acquisition du chloroplaste par endosymbiose secondaire.

En revanche chez les coccolithophores l'observation de capacités mixotrophiques n'a été constatée que pour la phase haploïde de *C.braarudii* (Houdan et al., 2006). Notre étude a montré que les phases haploïdes, mais pas les phases diploïdes, des coccolithophores *E.huxelyi* et *Jomonlithus* sp. ont la capacité de phagocyter des bactéries (Fig. 18) tout en maintenant une activité photosynthétique. Les phases haploïdes de ces espèces de coccolithophore ont donc des stratégies de nutritions mixotrophes. Ainsi, d'une manière générale, les phases haploïdes des coccolithophores pourraient avoir un double rôle dans la boucle microbienne en étant non seulement des producteurs primaires mais aussi des micro-brouteurs participant à la régulation des populations microbiennes et au recyclage de la matière organique.

Selon Valero et al le maintien d'un cycle de vie haplo-diploïde serait seulement possible quand chaque stade du cycle de vie explore des niches distinctes (Mable et Otto, 1998, Valero et al., 1992). La mixotrophie permet d'élargir les ressources nutritives disponibles ainsi que les niches écologiques exploitables. L'existence de mixotrophie dans l'un des stades pourrait donc en partie expliquer le maintien des deux phases dans le cycle de vie de ces espèces.

V Conclusion

Toutes les expériences réalisées au cours de cette étude démontrent que les phases haploïde et diploïde d'*Emiliania huxleyi* présentent des physiologies et des écologies différentielles.

La phase diploïde non-motile est comparativement très compétitive dans des conditions pauvres en nutriments et sous de fortes intensités lumineuses. Ces propriétés physicochimiques caractérisent des masses d'eaux stratifiées et fortement irradiées en surface, connues pour être propice au développement des efflorescences diploïdes calcifiantes.

La phase haploïde, sujette a la photoinhibition et moins performante pour l'acquisition des nitrates, pourrait se propager soit juste après l'efflorescence lorsque la calcite flottante assombri les eaux de surface et que la dégradation bactérienne des cellules diploïdes charge l'écosystème en matière organique. Le caractère mixotrophe du stade haploïde permettrait alors l'exploitation de ces conditions riches en bactéries et nutriment, et sans competition avec la phase diploide. De plus, la phase haploïde, qui peut se déplacer a l'aide de ses flagelles, pourrait prolonger son existence dans des eaux plus profondes, plus riches en nutriments, et a faible intensite lumineuse, typiquement au niveau du « Deep Chlorophyll Maximum ».

Cette étude démontre donc que chaque phase du cycle de vie chez *E. huxleyi* a un role fondamental au sein de cette espèce. Les donnes suggèrent que le cycle de vie haplo-diploide est une stratégie clef d'adaptation à un environnement instable et très dynamique, non seulemement chez *E. huxleyi* mais probablement pour l'ensemble des coccolithophores. Il apparait désormais nécessaire d'aller vérifier ces hypothèses dans le milieu naturel, avec des méthodes de marquages moléculaire (type COD-FISH, Frada et al. 2006).

VI Bibliographie

Alonso-Saez, L., Balagué, V., Sà, E.L., Sánchez, J., J.Pinhassi, J., R. Massana, R., J.Pernthaler, J., Pedrós-Alió, C.,Gasol, J.M., in press. Seasonality in bacterial diversity in north-west Mediterranean coastal waters: assessment through clone libraries, fingerprinting and FISH.

Aminot, A.,Kérouel, R., 2004. Dissolved organic carbon, nitrogen and phosphorus in the N-E Atlantic and the N-W Mediterranean with particular reference to non-refractory fractions and degradation. Deep Sea Research I 51, 1975-1999

Andreae, M.O.,Crutzen, P.J., 1997. Atmospheric aerosols: Biogeochemical sources and role in atmospheric chemistry. Science
276, 1052-1056

Benschneider, K.,Robinson, R.J., 1952. A new spectrophotometric method for the detection of nitrate in seawater. J Mar Res 11, 87–96.

Billard, C., 1994. Life Cycles, The Haptophyta Algae. Clarendon Press, Oxford, 167-186

Billard, C.,Inouye, I., 2004. What's new in coccolithophore biology? Coccolithophores - From molecular processes to global impact. SpringerVerlag, New York, Berlin, Heidelberg, London, Paris, Tokyo,

Bratbak, G., Egge, J.K.,Heldal, M., 1993. Viral mortality of the marine algae *Emiliania huxleyi* (Haptophyceae) and the termination of the algal bloom. Marine Ecology Progress Series 93, 39-48

Brown, C.W.,Yoder, J.A., 1994. Distribution pattern of coccolithophorid blooms in the Western North Atlantic Ocean. Cont. Shelf Res. 14, 175-198.

Brussaard, C.P.D., 2004. Viral Control of Phytoplankton Populations - a Review. The Journal of Eukaryotic Microbiology 51, 125-138

Castberg, T., Larsen, A., Sandaa, R.A., Brussard, C.P.D., Egge, J.K., Heldal, M., Thyrhaug, R., van Hannen, E.J.,Bratbak, G., 2001. Microbial population dynamics and diversity during a bloom of the marine coccolithophorid Emiliania huxleyi (Haptophyta). Marine Ecology Progress Series 221, 39-46

Charlson, R.J., Lovelock, J.E., Andreae, M.O.,Warren, S.G., 1987. Oceanic phytoplankton, atmospheric sulphur, cloud albedo and climate. Nature 326, 655 - 661

De Vargas, C.,Probert, I., in press. Origin and Evolution of Coccolithophores: From Coastal Hunters to Oceanic Farmers, Evolution of Aquatic Photoautotrophs. Academic Press,

Edvardsen, B., Eikrem, W., Green, J.C., Andersen, R.A., Moon-van der Staay, S.Y.,Medlin, L., K., 2000. Phylogentic reconstruction of the Haptophyta inferred from the 18S ribosomal DNA sequences and available morphological data. Phycologia 39, 19-35

Egge, J.K.,Heimdal, B.R., 1994. Blooms of phytoplankton including Emiliania huxleyi (Haptophyta).Effects of nutrient supply in different N: P ratios. Sarsia 79, 333–348.

Falkowski, P.G., Katz, M.E., Knoll, A.H., Quigg, A., Raven, J.A., Schofield, O.,Taylor, F.J.R., 2004. The Evolution of Modern Eukaryotic Phytoplankton. Science 305, 354-360

Flynn, K.J., 2003. Modelling muli–nutrient interactions in phytoplankton:balancing simplicity and realism. Prog. Oceanogr.
56, 249–279.

Frada, M., Not, F., Probert, I.,de Vargas, C., 2006. $CaCO_3$ Optical Detection With Fluorescent In Situ Hybridazion: A New Method to identify and Quantify calcifyng Microorganisms From the Oceans. Journal of Phycology 42, 1162-1169

Green, J.C.,Hori, K., 1994. Flagella and flagellar roots, The Haptophyte Algae. Oxford, 91-110.

Harper, J.L., 1977. Population of Biology of Plants.

Harris, G.N., Scanlan, D.J.,Geider, R.J., 2005. Acclimation of *Emiliania Huxleyi* (Primnesiophyceae) to photon flux density. J. Phycol. 41, 851–862

Holligan, P.M., Fernández, E., Aiken, J., Balch, W.M., Boyd, P., Burkill, P.H., Finch, M., Groom, S.B., Malin, G., Muller, K., D.A.Purdie, C.Robinson, C.C.Trees, S.M.Turner,Wal, P.v.d., 1993. A biochemical study of the coccolithophore, Emiliania huxleyi, in the north Atlantic. Global Biogeochemical Cycles 7, 879-900

Holligan, P.M., Fernández, E., Aiken, J., MBalch, W., Boyd, P., Burkill, P.H., M. Finch, S.B. Groom, Malin, G., Kuller, M., Purdie, D.A., Robinson, C., C.C.Trees, Turner, S.M.,Wal, P.v.d., 1993. A biochemical study of the coccolithophore, Emiliania huxleyi, in the north Atlantic. Global Biogeochemical Cycles 7, 879-900.

Honjo, S.F., 1996. Fluxes of particles to the interior of the open oceans, Particles Flux in the Ocean. SCOPE 57, New York, 91-154.

Houdan, A., Billard, C., Marie, D., Not, F., Saez, A., Young, G.,Probert, I., 2004. Holococcolithophores-heterococcolithophores (Haptophyta) life cycles: flow cytometry analysis of relative ploidy levels. Systematics and Biodiversity. 1, 453-465

Houdan, A., Probert, I., Van Lenning, K.,Lefebvre, S., 2005. Comparison of photosynthetic responses in diploid and haploid life-cycle phases of Emiliania huxleyi (Prymnesiophyceae). Marine Ecology Progress Series

Houdan, A., Probert, I., Zatylny, C., Véron, B.,Billard, C., 2006. Ecology of Oceanic Coccolithophores. I. Nutritional Preferences of the Two Stages in the Life Cycle of *Coccolithus braarudii* and *Calcidiscus leptoporus*. AQUATIC MICROBIAL ECOLOGY 44, 291-301

Inouye, I.,Kawachi, M., 1994. The haptonema, The Haptophyte Algae. Clarendon Press, Oxford, 73-90.

Jacquet, S., Heldal, M., Iglesias-Rodriguez, D., Larsen, A., Wilson, W.,Bratbak, G., 2002. Flow cytometric analysis of an Emiliana huxleyi bloom terminated by viral infection. AQUATIC MICROBIAL ECOLOGY 27, 111-124

Jones, H.L.J., Leadbeater, B.S.C.,Green, J.C., 1994. Mixotrophy in haptophytes, The Haptophyte Algae. For the Systematics Association by Clarendon Press, Oxford, 247-264.

Jordan, R.W.,Chamberlain, A.H.L., 1997. Biodiversity among haptophyte algae. Biodiversity and Conservation 6, 131-152

Keller, M.D., Selvin, R.C., Claus, W.,Guillard, R.R.L., 1987. Media for the culture of oceanic phytoplankton. Journal of Phycology 23, 633-638

Koroleff, F., 1969. Determination of total nitrogen, phosporus and iron in fresh water by photo-oxidation with ultraviolet radiation. Int. Counc. Explor. Sea 8,

Lotka, A.J., 1934. Théorie analytique des associations biologiques. Actualités scientifiques

Mable, B.K.,Otto, S.P., 1998. The evolution of life cycles with haploid and diploid phases. BioEssays 20, 453-462

Malin, G.,Steinke, M., 2004. Dimethyl Sulfide Production: What is the Contribution of the Coccolithophores? Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo,

Murphy, J.,Riley, J.P., 1962. A modified single solution method for the determination of phosphate in naturals waters. Anal. Chim. Acta. 27, 31-36

Nielsen, M.V., 1997. Growth, dark respiration and photosynthetic parameters of the coccolithophorid Emiliania huxleyi (Prymnesiophyceae) acclimated to different day length-irradiance combinations. J. Phycol 33, 818–822

Okada, H.,McIntyre, A., 1979. Seasonal distribution of modern coccolithophores in the western north atlantic ocean. Marine Biology 54, 319-328

Paasche, E., 2001. A review of the coccolithophorid Emiliania huxleyi (Prymnesiophyceae), with particular reference to growth, coccolith formation, and calcificationphotosynthesis interactions. Phycologia 40, 503–529

Riegman, R., Noordeoos, A.A.,CedeI, G.C., 1992. Phaeocystis blooms and eutrophication of the continental coastal zones of the North Sea. Mar. Biol. 112, 479–484.

Riegman, R., Stolte, W., Noordeloos, A.A.M.,Slezak, D., 2000. Nutrient uptake and alkaline phosphatase (ec 3:1:3:1) activity of emiliania huxleyi (prymnesiophyceae) during growth under n and p limitation in continuous cultures. Journal of Phycology 36, 87-96

Rippka, R., Coursin, T., Hess, W., Lichtlé, C., Scanlan, D.J., Palinska, K.A., Iteman, I., Partensky, F., Houmard, J.,Herdman, M., 2000. *Prochlorococcus marinus* Chisholm et al. 1992 subsp. *pastoris* subsp. nov. strain PCC 9511, the first axenic chlorophyll a_2/b_2-containing cyanobacterium (Oxyphotobacteria). International Journal of Systematic and Evolutionary Microbiology 50, 1833-1847

Rost, B., Riebesell, U., Burkhardt, S.,Sultemeyer, D., 2003. Carbon acquisition of bloom-forming marine phytoplankton. Limnology and Oceanography 48, 55-67

Smayda, T.J., 1997. Bloom dynamics: Physiology, behavior, trophic effects. Limnol.Oceanograf. 42, 1132-1136

Stolte, W., T.McCollin,Noordeloos, A.A.M., 1994. Effect of nitrogen source on the size distribution within marine phytoplankton populations. J. Mar. Exp. Biol. Ecol. 184, 83–97

Strickmand, J.D.,Parson, T.R., 1972. A practical handbook of seawater analysis. Bull. Fish. Res. 167,

Tréger, P.,LeCorre, P., 1975. Book Manuel d'analyse des sels nutritifs dans l'eau de mer (utilisation de l'autoAnalyseur Technicon II). Laboratoire d'Océanographie Chimique de l'Université de Bretagne Occidentale., Pages

Tyrrel, T.,Taylor, A.H., 1996. A modelling study of Emiliania huxleyi in the NE Atlantic. J. Marine Syst. 9, 83-112.

Tyrrell, T.,Merico, A., 2004. Emiliania huxleyi: Bloom Observation and the Conditions that Induce Them, Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo,

Valero, M., Richerd, S.,Perrot, V., 1992. Evolution of Alternation of Haploid and Diploid Phases in Life Cycles. TRENDS in Ecology and Evolution 7, 25-29

Volterra, V., 1926. Fluctuations in the abundance of a species considered mathematically. Nature 118, 558–560

Wood, E.D., Armstrong, F.A.J.,Richards, F.A., 1967. Determination of nitrate in sea water by cadmium copper reduction to nitrite. J. Mar. Biol. Ass. U.K. 47, 23-31

Young, J.R., 1994. Functions of coccoliths., Coccolithophores. Cambridge, UK, 63-82

ANNEX 5

A newfound ancient diversity of protists dominates photosynthesis in open oceans

Hui Liu[1,2], Ian Probert[1], Julia Uitz[3], Hervé Claustre[4], Stéphane Aris-Brosou[5], Miguel Frada[2], Fabrice Not[2], Colomban de Vargas[1, 2]

[1] *CNRS, UMR 7144 & UPMC Univ Paris 06, Equipe EPPO - Evolution du Plancton et Paléo-Océans, Station Biologique de Roscoff, 29682, France.*

[2] *Institute of Marine and Coastal Sciences, Rutgers University, New Brunswick, NJ 08901, U.S.A.*

[3]*Marine Physical Laboratory, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093-0238, U.S.A.*

[4] *CNRS, UMR 7093 & UPMC Univ Paris 06, Laboratoire d'Océanographie de Villefranche/Mer, 06234, France.*

[5]*Department of Biology and Department of Mathematics and Statistics, University of Ottawa, Ottawa, K1N 6N5, Canada*

Over the last twenty years a dogma has become established that cyanobacteria, which evolved oxygenic photosynthesis more than three billion years ago, are still the major light harvesters driving primary productivity in open oceans[1]. Here we show that tiny (<3µm) unicellular eukaryotes belonging to the haptophyte lineage are dramatically diverse in the planktonic photic realm, where they appear to dominate photoreception. The use of Haptophyta-specific primers and PCR conditions adapted for GC-rich genomes circumvented biases inherent in classical genetic approaches to exploring environmental eukaryotic biodiversity, and led to the discovery of hundreds of novel haptophyte taxa in five clone libraries from sub-polar and sub-tropical oceanic waters. Phylogenetic analyses suggest that this

diversity emerged in Paleozoic oceans, thrived and diversified in the permanently oxygenated Mesozoic Panthalassa, and currently comprises thousands of ribotypic species, mostly belonging to low-abundance populations of the 'rare biosphere'[2]. This extreme biodiversity correlates to the pervasive presence in the photic zone of the world ocean of *19'-hexanoyloxyfucoxanthin* (*Hex*), a photosynthetic pigment found exclusively in chloroplasts of haptophyte origin[3]. Our new estimates of depth-integrated relative abundance of *Hex* suggest that haptophytes actually dominate the photo-reception standing stock in modern oceans. Their ecological success, arguably based on mixotrophy and relatively complex morphology, may have significantly impacted the biological pump. These results add to the growing evidence that eukaryogenesis and protistan evolution[4] are key forces in the functioning of the biosphere.

Oxygenic photosynthesis, the most complex and energetically powerful molecular process in biology, was invented by cyanobacteria more than three billion years ago in Archean oceans. Marine photosynthesis still contributes ~50% of total primary production on Earth[5]. This revolutionary process was integrated, at least once, into an ancestral eukaryotic lineage through the evolution of chloroplasts, which themselves were redistributed to a large variety of aquatic eukaryote lineages via permanent secondary and tertiary endosymbioses[6]. Despite this evolutionary trend from photosynthetic prokaryotes to eukaryotes, particularly visible in today's coastal oceans where microalgae such as diatoms and dinoflagellates are omnipresent, cyanobacteria have been repeatedly claimed as the champions of photosynthesis in open ocean waters[7]. This followed the introduction of flow cytometry and molecular genetic approaches to biological oceanography in the 1980s which revealed astonishing concentrations of cyanobacteria from the genera *Prochlorococcus* and *Synechococcus* in marine waters. The physiology, ecology, and functional and environmental genomics of these prokaryotes are subjects of ongoing intensive study [e.g.8].

Several lines of evidence in fact argue for eukaryotic supremacy over marine oxygenic photosynthesis. Flow cytometric cell counts[e.g.9] consistently show that picophototrophic protists (0.2-3μm) are indeed 1-2 orders of magnitude less abundant than cyanobacteria. However, biophysical and group-specific ^{14}C-uptake measurements suggest that tiny eukaryotes could in fact, through equivalent or higher growth rates of relatively larger cells, dominate carbon biomass and net production in both coastal[10] and oceanic[9] settings. HPLC analyses of group-specific accessory pigments have further stressed the ecological prevalence of phototrophic protist taxa. In particular, *Hex* was originally found to account for 20-50% of total chlorophyll-a (Chl*a*) biomass in tropical Atlantic and Pacific regions[11] and has since been consistently reported in open ocean photic-zone waters[e.g.17,12], suggesting a ubiquitous distribution of haptophytes in upper layers of the water column. Surveys of genetic diversity based on environmental SSU rDNA libraries over the last decade have unveiled an unexpected diversity of tiny eukaryotes in all oceans [e.g.13]. Paradoxically, most picoeukaryotic sequence diversity from photic layers represented novel *heterotrophic*[14] and *parasitic*[15] protists within phyla traditionally thought to be dominated by photoautotrophs. This suggested that marine protist diversity might be significantly skewed towards heterotrophic taxa[16], as appears to be the case in the prokaryotic world. However, the paucity of haptophyte nuclear rDNA sequences in these surveys contrasts strikingly with the abundance of *Hex* in marine waters.

Here we show that previous nuclear rDNA PCR-based studies of eukaryotic communities were subject to severe selective amplification biases. Several groups of eukaryotes known to have long and/or GC rich rDNA are virtually missing from environmental clone libraries produced by classical PCR amplification protocols using 'general eukaryote' SSU rDNA primers[eg.13,14]. This is the case for the haptophytes, the rDNA of which has a mean GC content of ~57%. We thus used haptophyte-specific primers and a PCR protocol designed for GC-rich genomes to amplify the LSU rDNA

D1-D2 fragments from picoplanktonic total-DNA extractions of seawater collected at 4 offshore stations in the Arctic and Indian oceans (Fig. S1, Table S1). Standard eukaryotic rDNA analyses of these samples yielded ~0.4-0.7% haptophyte sequences[12, 17]. In contrast, our data reveal hundreds of novel rDNA sequences from tiny haptophytes. Rarefaction curves for individual clone libraries (Fig. 1) indicate that current sequencing effort is far from exhaustive, notably in sub-tropical waters where genetic diversity is particularly dramatic. Estimates of the quantities of unique ribotypes using the Chao1 estimator were 1098-1147 and 325-509 respectively for the Indian and Arctic ocean samples (Table S2). The frequency distribution of unique ribotypes (Fig. 1) indicates higher species richness in sub-tropical waters, with a substantial number of orphan and deep-branching genotypes in both warm and cold waters. This parallels recent observations of a 'rare biosphere' or 'seed bank' of ancient and rare taxa in the marine prokaryotic world[2].

The taxonomy and evolutionary history of the novel environmental sequences were inferred from phylogenetic reconstructions including 64 LSU rDNA sequences from clonal culture strains representing a cross-section of described haptophyte biodiversity. All novel environmental sequences belonged to the Haptophyta (Fig. 2), a division of chlorophyll a+c containing eukaryotes classically considered as nannoplankton (3-20µm) and including the calcifying coccolithophores[18]. However, not a single environmental sequence was strictly identical to any of the taxonomically defined sequences. The vast majority of environmental sequences form new clusters branching deep in the haptophyte phylogeny, most being related to *Chrysochromulina* species from clade-B2 within the order Prymnesiales[19]. The described representatives of this clade are nannoplanktonic and known almost exclusively from coastal and shelf environments[20]; our data suggest these are derived from open ocean picoplanktonic taxa (Fig. 2). Note that other new groups of picohaptophytes appear to be ancestors of the two other major prymnesiophyte lineages, the Phaeocystales and the Calcihaptophycidae[18]. Calibration of our tree with the

stratigraphic record of coccolithophore taxa (Fig. 2) suggests that tiny haptophyte biodiversity emerged in Paleozoic oceans (Fig. S2), before the evolution of intracellular biomineralization in the Calcihaptophycidae, which according to both fossil and molecular clock data occurred ~220 Ma[18]. The phylogenetic depth of most picohaptophyte clusters argues for a Mesozoic diversification of the group which may have thrived in the newly permanently oxygenated and largely oligotrophic Panthalassic ocean, conditions which served as a selection matrix for a wide range of chlorophyll a+c containing protists[4]. Many genotypes or genotype clusters were found exclusively in either sub-arctic or sub-tropical oceans, supporting lineage partitioning between cold mixed and warmer stratified waters (Fig. 3). The phylogeographic distribution of genetic types suggests that tropical waters were the original centre of diversification, with biodiversity spreading secondarily into higher latitudes, a scenario that fits the putative early radiation of the group in the warm Panthalassa.

Our genetic survey positions the novel haptophytes as the *most diverse* group of picoautotrophs in modern open oceans. Recent exploration of *chloroplastic* SSU rDNA in pelagic[21] and coastal[22] environments supports this conclusion. Chloroplastic genomes are typically not GC-biased and thus accessible using standard PCR protocols. Haptophytes dominate the emerging chloroplastic view of marine tiny eukaryotic phytoplankton in terms of both diversity and abundance. In a year-round dataset from the Gulf of Naples[22], >45% total and >70% novel chloroplastic rDNA sequences were of haptophyte origin, 55% of them from Prymnesiales clade-B2 (Fig. S3). This extreme haptophyte diversity coincides with a *numerical* significance among oceanic eukaryotes. Group-specific FISH data from various oceanic settings indicate that haptophytes represent up to 35% of total picoeukaryotic cell numbers[23]. Dot blot hybridizations of specific eukaryotic chloroplastic rDNA confirmed an overall dominance (mean of ~45%) of haptophytes over a 2-year survey of ultra-phytoplankton (<5µm) in Mediterranean waters[22]. In order to assess whether these observations are representative over a broad spatio-temporal

scale, we evaluated the contribution of haptophytes to global oceanic phototrophic biomass using an empirical model based on >2400 worldwide vertical profiles of HPLC pigment data combined with monthly ocean-colour composites of surface Chl*a* concentrations measured by the SeaWiFS satellite in the year 2000 (Fig. 4). This analysis revealed that *Hex* is the dominant component of global oceanic photosynthetic biomass, representing about twice the Chl*a* standing stocks of either oceanic diatoms or prokaryotes. Haptophytes appear thus to represent the *background* oceanic light harvesters, contributing from 30-50% of photoreception standing stock across the world ocean.

The remarkable diversity of tiny haptophytes reflects their flexibility to adapt to diverse ecological niches, resulting in competitive superiority over other photosynthetic groups in most oceanic settings, notably over vertical scales. Significant *Hex* concentrations were recorded in 200-300m deep layers of the clearest waters on Earth in the South Pacific gyre[24], depths where irradiance at noon is not even sufficient for photosynthesis to cover basic cellular metabolic requirements. The ecological success of picohaptophyte diversity may thus rely on a fundamental eukaryotic feature: phagocytosis. Haptophytes, notably members of the Prymnesiales, can capture prey with their third flagellum-like appendix, the *haptonema*, and ingest organic matter by phagocytosis[25,26]. Mixotrophy, the intricate combination of cyanobacterial photosynthesis and eukaryotic phagocytosis, is arguably the key character of most oceanic eukaryotic phytoplankton, allowing them to reach relatively large size while maintaining prokaryote-like growth rates, and to radiate into a wide diversity of ecogenotypes exploiting many different pathways of organic matter synthesis. Complex mixotrophic regimes may also explain why none of the open ocean haptophytes are currently available in culture collections.

Finally, tiny haptophytes display morphological features that suggest they play

critical roles in both organic carbon synthesis and burial on a global scale. Size analyses of cells identified by haptophyte-specific fluorescent probes revealed a mode of ~4μm, with largest sizes of 8-9μm (Fig. S4 and Table S4). In terms of volume, haptophytes are thus typically 300-3000 times bigger than *Procholorococcus*, the most abundant marine cyanobacteria. The few available EM images (Fig. S5) indicate that picohaptophytes do produce organic plate scales, a plesiomorphic character common to the overwhelming majority of prymnesiophytes. Abundant and diverse *Chrysochromulina* spp. scales were recently observed in Atlantic surface sediments collected at 4850m[27]. The taxonomic origin and pristine preservation of these scales, previously overlooked in deep-sea sediments due to their minute size (~1μm), suggest that they were rapidly transported to the sea-floor, most likely by aggregation to sinking biological material. Eukaryotic scales made of proteins embedded into cellulose and other polysaccharides potentially provide abundant resistant and sticky matter to enhance aggregation and flux of marine snow particles to the deep ocean, contributing to the largely underestimated role of coagulation of small phytoplankters in the biological pump[28]. Besides their unanticipated diversity and abundance, the tiny haptophytes may have been essential mediators of carbon fluxes from the atmosphere to the deep oceans and the lithosphere throughout much of the Phanerozoic Eon.

METHODS SUMMARY

Genomic DNA was extracted from the 0.2-3μm fraction of five seawater samples collected in the Arctic and Indian oceans (Fig. S1) as well as from clonal haptophyte cultures. 28S Partial LSU rDNA was amplified with a forward Haptophyta-specific and reverse general eukaryote primer set, using a PCR protocol suitable for GC-rich DNA. Clone libraries were constructed for environmental samples and ~200 LSU rDNA clones randomly selected and sequenced per library. Sequences were stringently screened for chimeras and a total of 674 environmental and 52 culture sequences were deposited in GenBank. Pairwise maximum likelihood distance matrices were used to estimate

rarefaction curves and species richness based on the average neighbour algorithm in DOTUR. Phylogenies were reconstructed with various methods and divergence times estimated by Bayesian analysis. Relaxed clock analyses were calibrated with five divergence dates from stratigraphic data. The global contribution of haptophytes to oceanic Chl*a* standing-stock was calculated using an empirical model, based on analysis of >2400 worldwide HPLC-derived pigment profiles, for estimating water column-integrated contents of Chl*a* and accessory pigments from surface Chl*a* concentration[29]. The model was adapted here to 19'-hexanoyloxyfucoxanthin (Hex), fucoxanthin (Fuco) and zeaxanthin (Zea), the diagnostic carotenoids of haptophytes, diatoms, and prokaryotes, respectively, and applied to monthly composites of SeaWiFS-derived surface Chl*a* concentrations from the year 2000, yielding Hex, Fuco and Zea contents integrated over the euphotic zone. The monthly values were converted into Chl*a* equivalents, summed to give annual values, and normalized to the annual mean euphotic layer-integrated Chl*a* content to determine the relative contribution of each group to total phytoplankton biomass (Fig. 4). For each group, an annual mean global Chl*a* standing-stock was calculated as the sum of the annual value for each pixel multiplied by pixel surface area. Detailed methods are available in the Supplementary Information section.

References

1. Chisholm, S.W. *et al.* A novel free-living prochlorophyte abundant in the oceanic euphotic zone. *Nature* **334**, 340-343 (1988).

2. Sogin, M.L. *et al.* Microbial diversity in the deep sea and the underexplored "rare biosphere". *PNAS* **103**, 12115-12120 (2006).

3. Van Lenning, K., Probert, I., Latasa, M., Estrada, M. & Young, J.R. Pigment diversity of coccolithophores in relation to taxonomy, phylogeny and ecological preferences, in *Coccolithophores, from Molecular Processes to Global Impact*. (eds. H.R. Thierstein & J.R. Young) 51-74 (Springer, New York; 2005).

4. Falkowski, P.G. *et al.* The Evolution of Modern Eukaryotic Phytoplankton. *Science* **305**, 354-360 (2004).

5. Field, C.B., Behrenfeld, M.J., Randerson, J.T. & Falkowski, P. Primary Production of the Biosphere: Integrating Terrestrial and Oceanic Components. *Science* **281**, 237-240 (1998).

6. Falkowski, P.G. & Knoll, A.H. *Evolution of primary producers in the sea*. (Elsevier Academic Press, 2007).

7. Goericke, R. & Welschmeyer, N.A. The marine prochlorophyte Prochlorococcus contributes significantly to phytoplankton biomass and primary production in the Sargasso Sea. *Deep Sea Research Part I: Oceanographic Research Papers* **40**, 2283-2294 (1993).

8. Kettler, G.C. *et al.* Patterns and Implications of Gene Gain and Loss in the Evolution of Prochlorococcus. *PLoS Genetics* **3**, e231 (2007).

9. Li, W.K.W. Composition of ultraphytoplankton in the central North Atlantic. *Marine Ecology - Progress Series* **122**, 1-8 (1995).

10. Worden, A.Z., Nolan, J.K. & Palenik, B. Assessing the dynamics and ecology of marine picophytoplankton: The importance of the eukaryotic component. *Limnology and Oceanography* **49**, 168-179 (2004).

11. Andersen, R.A., Bidigare, R.R., Keller, M.D. & Latasa, M. A comparison of HPLC pigment signatures and electron microscopic observations for oligotrophic waters of the North Atlantic and Pacific Oceans *Deep Sea Research Part II: Topical Studies in Oceanography* **43**, 517-537 (1996).

12. Not, F. *et al.* Phytoplankton diversity across the Indian Ocean: a focus on picoeukaryotes. *Deep Sea Research Part I: Oceanographic Research Papers* (in press).

13. Moon-van der Staay, S.Y., De Wachter, R. & Vaulot, D. Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. *Nature* **409**, 607-610 (2001).

14. Massana, R. *et al.* Phylogenetic and Ecological Analysis of Novel Marine Stramenopiles. *Appl. Environ. Microbiol* **70**, 3528-3534 (2004).

15. Guillou, L. *et al.* Widespread occurrence and genetic diversity of marine parasitoids belonging to Syndiniales (Alveolata). . *Environmental Microbiology* (in press).

16. Vaulot, D., Romari, K. & Not, F. Are autotrophs less diverse than heterotrophs in marine picoplankton? *Trends in Microbiology* **10**, 266-267 (2002).

17. Lovejoy, C., Massana, R. & Pedros-Alio, C. Diversity and Distribution of Marine Microbial Eukaryotes in the Arctic Ocean and Adjacent Seas. *Appl. Environ. Microbiol.* **72**, 3085-3095 (2006).

18. de Vargas, C., Aubry, M.P., Probert, I. & Young, J. The Origin and Evolution of Coccolithophores: Form Coastal Hunters to Oceanic Farmers., in *Evolution of Primary Producers in the Sea*. (eds. P.G. Falkowski & A.H. Knoll) 251-286 (Elsevier Academic Press, Amsterdam, Boston, Heidelberg, London, New York, Oxford, Paris, San Diego, San Francisco, Singapore, Sydney, Tokyo; 2007).

19. Edvardsen, B. *et al.* Phylogenetic reconstruction of the Haptophyta inferred from 18S ribosomal DNA sequences and available morphological data. *Phycologia* **39**, 19-35 (2000).

20. Edvardsen, B. & Paasche, E. Bloom dynamics and physiology of Prymnesium and Chrysochromulina, in *Physiological ecology of harmful algal blooms*, Vol. 41. (eds. D.M. Anderson, A.D. Cembella & G.M. Hallegraeff) 193-208 (Springer, Berlin; 1998).

21. Fuller, N.J. *et al.* Molecular analysis of photosynthetic picoeukaryote community structure along an Arabian Sea transect. *Limnology and Oceanography* **51**, 2502-2514 (2006).

22. McDonald, S.M., Sarno, D., Scanlan, D.J. & Zingone, A. Genetic diversity of eukaryotic ultraphytoplankton in the Gulf of Naples during an annual cycle. *Aquatic Microbial Ecology* **50**, 75-89 (2007).

23. Worden, A.Z. & Not, F. Ecology and Diversity of Picoeukaryotes in *Microbial Ecology of the Oceans*. (ed. D.L. Kirchman) 159-205 (Wiley, Hoboken; 2008).

24. Ras, J., Claustre, H. & Uitz, J. Spatial variability of phytoplankton pigment distributions in the Subtropical South Pacific Ocean: comparison between in situ and predicted data. *Biogeosciences* **5**, 353-369 (2008).

25. Tillmann, U. Phagotrophy of a plastidic haptophyte, Prymnesium patelliferum. . *Aquat Microb Ecol* **14**, 155-160 (1998).

26. Kawachi, M., Inouye, I., Maeda, O. & Chihara, M. The haptonema as a food-capturing device: observations on Chrysochromulina hirta (Prymnesiophyceae). *Phycologia* **30**, 63-573 (1991).

27. Gooday, A.J., Esteban, G.F. & Clarke, K.J. Organic and siliceous protistan scales in north-east Atlantic abyssal sediments. *Journal of the Marine Biological Association of the United Kingdom* **86**, 679-688 (2006).

28. Richardson, T.L. & Jackson, G.A. Small Phytoplankton and Carbon Export from the Surface Ocean. *Science* **315**, 838-840 (2007).

29. Uitz, J., Claustre, H., Morel, A. & Hooker, S.B. Vertical distribution of phytoplankton communities in open ocean: An assessment based on surface chlorophyll. *Journal of Geophysical Research* **111**, C08005 (2006).

30. Bown, P.R. Calcareous nannoplankton evolution: a tale of two oceans. *Micropaleontology* **51**, 299-308 (2005).

Supplementary Information **is linked to the online version of the paper at** ww.nature.com/nature.

Acknowledgements Thanks to Swati Narayan-Yadav for practical help to obtain genetic data, Ramon Massana for sharing pico-DNA extractions, Jeremy Young for providing SEM images, and Dave Gruber for critical discussions. This work was supported by US NSF grants DEB-0415351 and IRES-0652093, an ATIP fellowship from the Centre National de la Recherche Scientifique (CdV), and the Canadian Natural Sciences and Engineering Research Council (SAB). It is part of the pluridisciplinary project BOOM (Biodiversity of Open Ocean Microcalcifiers), funded by the Institut Français de la Biodiversité via the Agence National de la Recherche, grant ANR-05-BDIV-004.

Author Contributions CdV conceived and designed the study; CdV and IP wrote the paper; HL obtained the genetic data and performed the phylogenetic analyses; HL, SAB, and CdV analyzed and interpreted the genetic data; IP isolated and/or maintained in culture all haptophyte strains; JU and HC developed and applied the statistical model for global pigment analysis; MF performed COD-FISH analyses to measure haptophyte cell size, and participated in DNA sequence acquisition; FN provided pico-planktonic total DNA samples and helped interpreting the results. All authors discussed the data, commented on the manuscript, and approved its final version.

Author Information DNA sequences were deposited in GenBank under accession numbers EU615823-EU616496, EU502871-EU502883 and EU729434-EU729479. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to C.d.V (Email: vargas@sb-roscoff.fr).

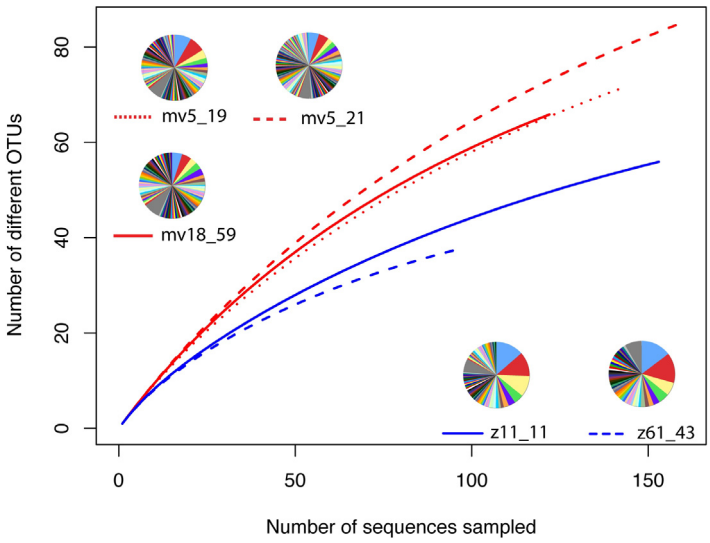
Figure Legends

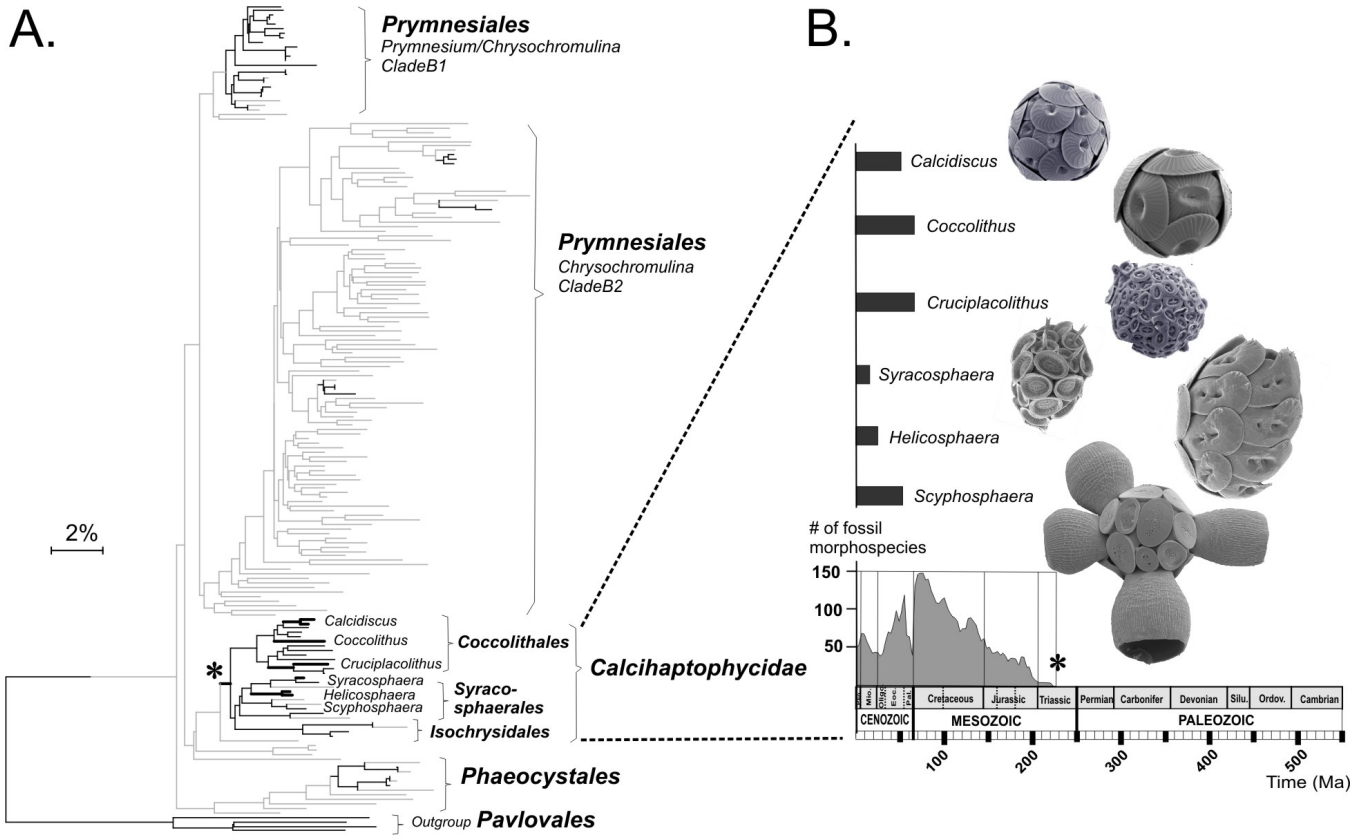
Figure 1 Rarefaction analysis for each environmental clone library based on unique 28S rDNAs (OTUs). Mv and z refer to Indian and North Atlantic ocean stations, respectively (Fig. S1) The pie charts indicate the amount of identical sequences in each retrieved OTU.

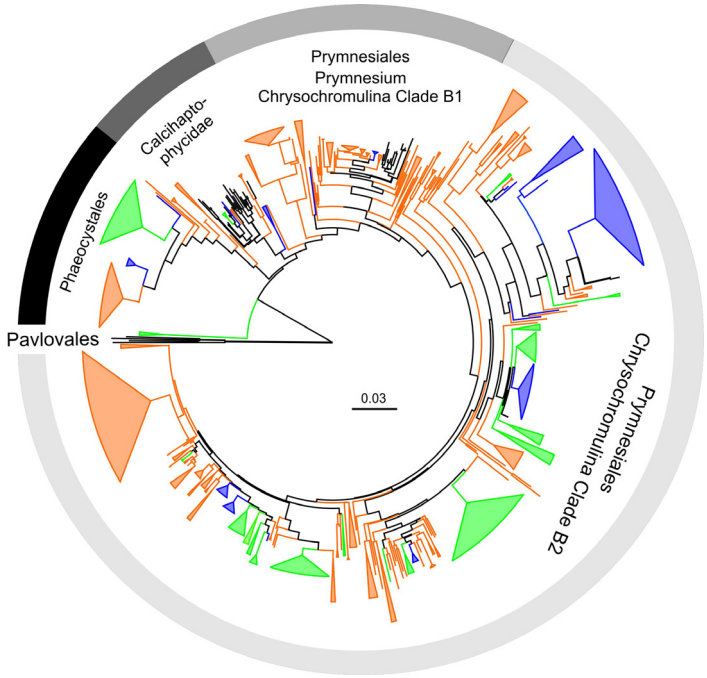
Figure 2 Phylogenetic assessment of the newfound haptophyte environmental diversity. A. LSU rDNA Neighbour Joining tree (5% divergence cutoff) including environmental sequences (grey branches) and a taxonomic cross-section of cultured haptophyte taxa (black branches, see Table S3 for species identification). B. Focus on the stratigraphic ranges (black rectangles) of key genera within the calcifying haptophytes[18] (thick black branches in the tree in A, and SEM images in B). The coccolithophore fossil record[30] (lower right) represents number of fossil morpho-species. Black clover symbols indicate the origin of haptophyte calcification ~220 Ma.

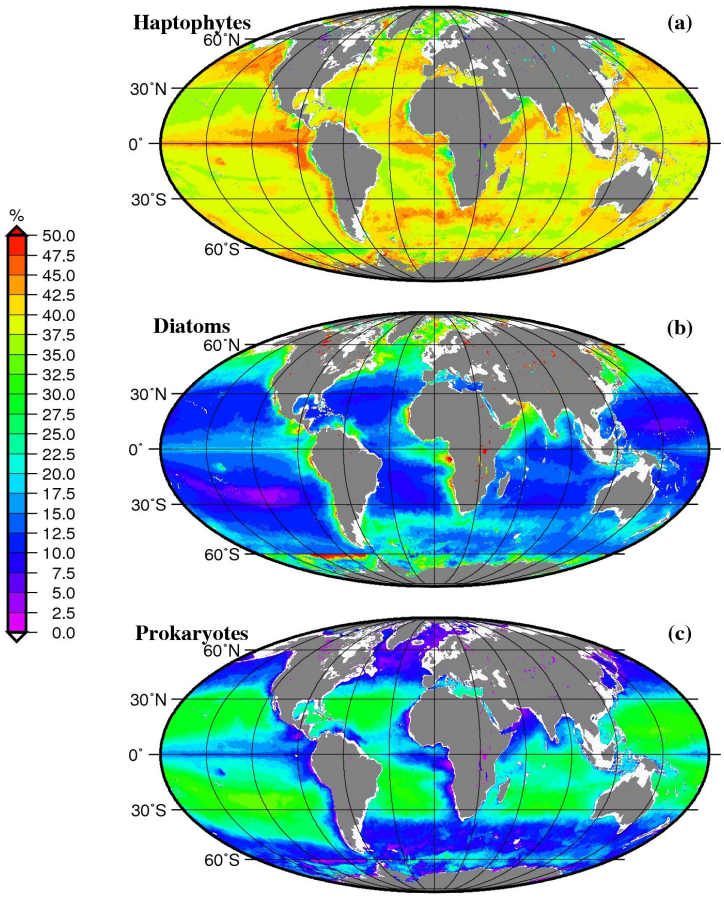
Figure 3 Biogeographic partitioning of environmental tiny haptophyte diversity. This Maximum Likelihood tree contains all 674 environmental LSU rDNA sequences with clustering above 97% similarity. Colour code: orange = sub-tropical; blue = sub-polar; green = both sub-polar and sub-tropical; black external branches = taxonomically-defined sequences from cultured haptophyte strains. Colour code applies to internal branches defining a strictly sub-tropical or sub-polar monophyletic group.

Figure 4 Relative contribution of (A) haptophytes, (B) diatoms, and (C) photosynthetic prokaryotes to total chlorophyll-*a* biomass in the photic layer of the world ocean over the year 2000. The average yearly standing stocks associated with these three groups are respectively 2.5×10^9, 1.3×10^9, and 1.1×10^9 kg Chl*a*. See methods sections for details of the calculation.


Number of different OTUs
0
20
40
60
80
0
50
100
150
Number of sequences sampled
mv5_19
mv5_21
mv18_59
z11_11
z61_43


A.
Prymnesiales
Prymnesium/Chrysochromulina
CladeB1
Prymnesiales
Chrysochromulina
CladeB2
2%
Calcidiscus
Coccolithus
Cruciplacolithus
Syracosphaera
Helicosphaera
Scyphosphaera
Coccolithales
Syraco-
sphaerales
Isochrysidales
Calcihaptophycidae
Phaeocystales
Outgroup
Pavlovales
B.
Calcidiscus
Coccolithus
Cruciplacolithus
Syracosphaera
Helicosphaera
Scyphosphaera
# of fossil
morphospecies
150
100
50
Mio.
Plio.
Eocc.
Paleo.
Cretaceous
Jurassic
Triassic
Permian
Carbonifer
Devonian
Silu.
Ordov.
Cambrian
CENOZOIC
MESOZOIC
PALEOZOIC
100
200
300
400
500
Time (Ma)


Prymnesiales
Prymnesium
Chrysochromulina Clade B1
Calcihapto-
phycidae
Phaeocystales
Pavlovales
0.03
Prymnesiales
Chrysochromulina Clade B2


Haptophytes
(a)
60°N
30°N
0°
30°S
60°S
Diatoms
(b)
60°N
30°N
0°
30°S
60°S
Prokaryotes
(c)
60°N
30°N
0°
30°S
60°S
%
50.0
47.5
45.0
42.5
40.0
37.5
35.0
32.5
30.0
27.5
25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0

Supplementary Information

A newfound ancient diversity of protists dominates photosynthesis in open oceans

Hui Liu, Ian Probert, Julia Uitz, Hervé Claustre, Stéphane Aris-Brosou, Miguel Frada, Fabrice Not, Colomban de Vargas

The file includes Supplementary Figures S1 to S5 with Legends, Supplementary Tables S1 to S4, Supplementary Methods, and additional references 1-22.

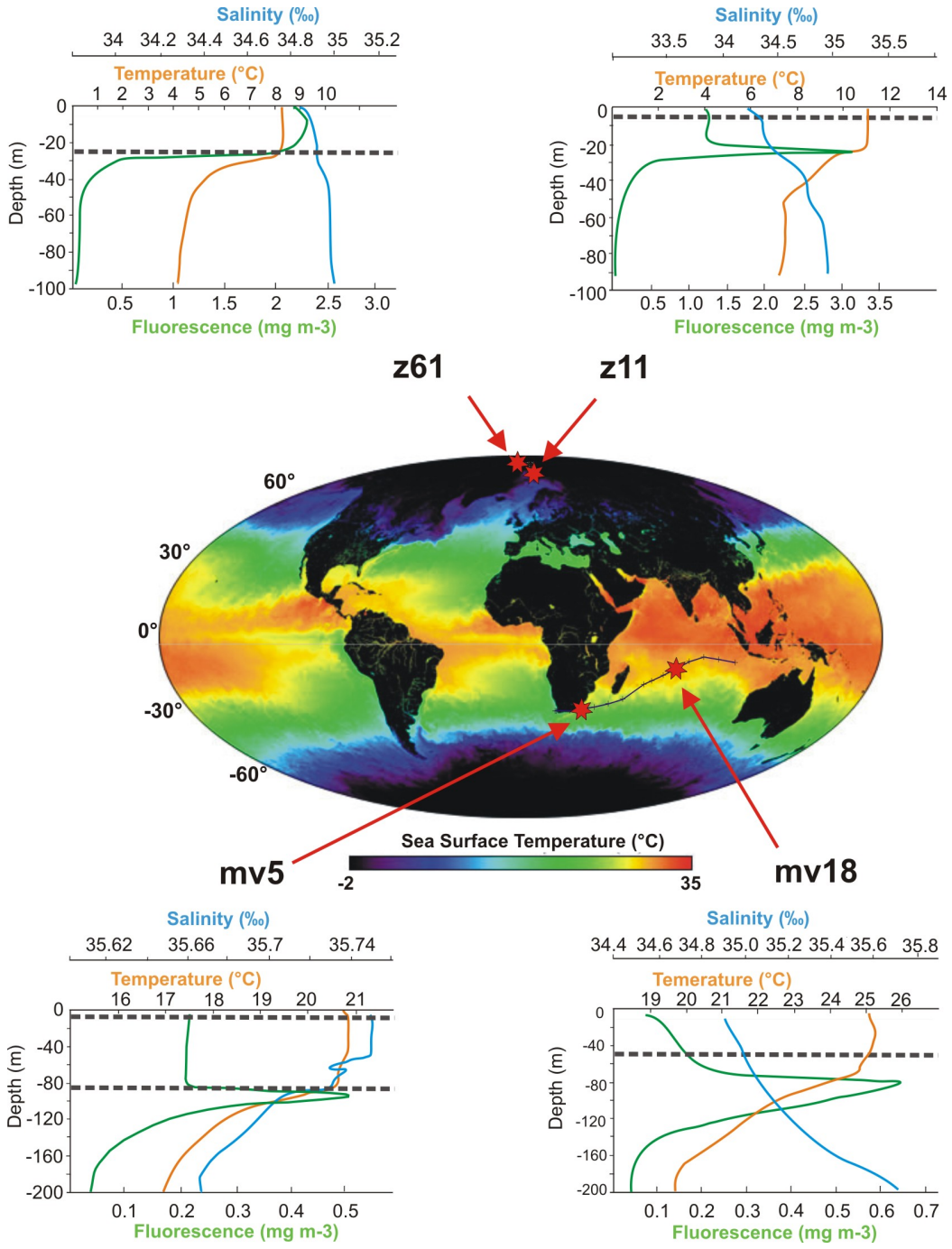


Figure S1 Cruise tracks and sampling locations. The four stations are marked with red stars. Temperature, salinity, and fluorescence profiles down to 100 or 200m depth are given for each station. Dotted lines indicate the depths at which water used for DNA extraction and rDNA sequencing was sampled with Niskin bottles. Global sea surface temperature corresponds to a monthly (May 2001) composite of data captured by the satellite *MODIS* (http://modis.gsfc.nasa.gov/). Further details showed in Table S1 below.

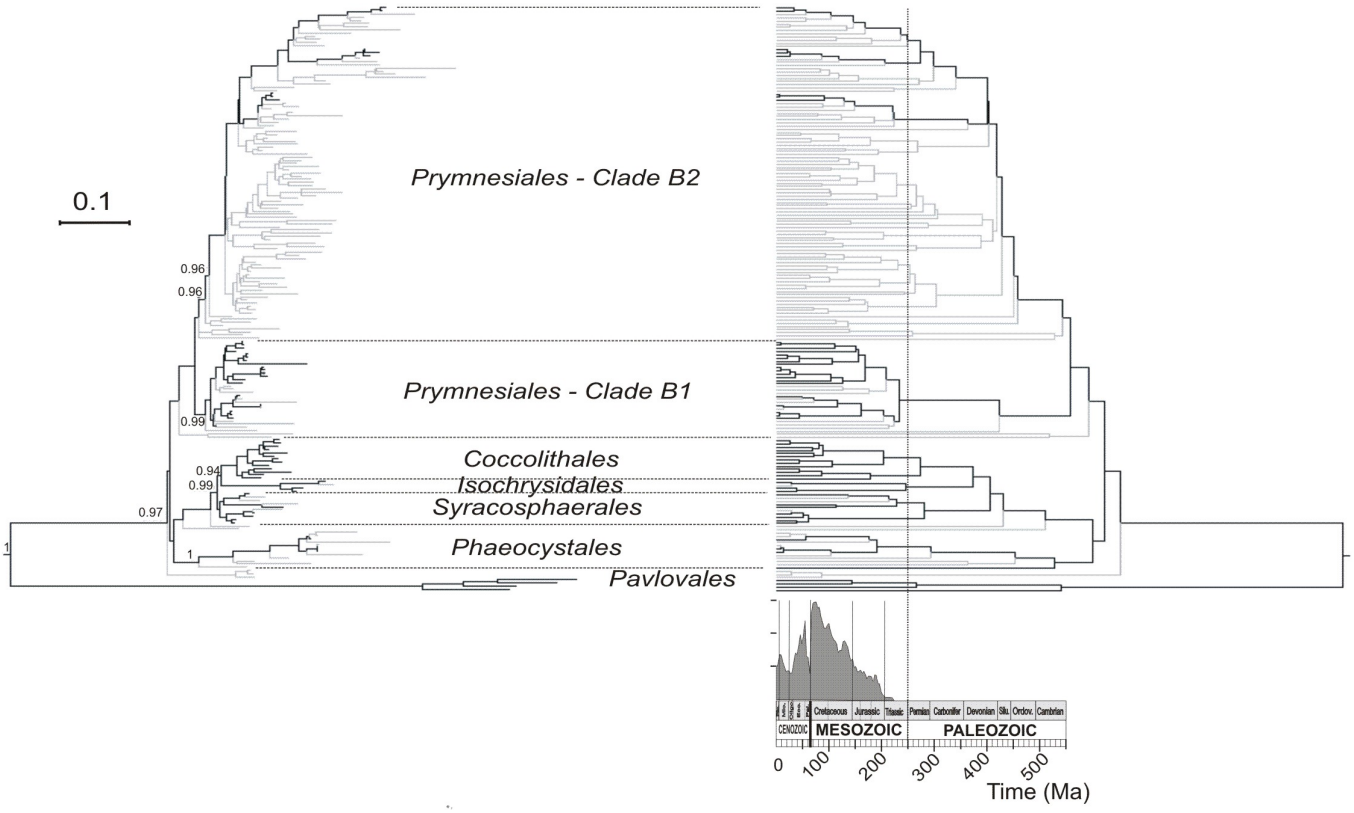


Figure S2 Diversification of haptophytes along geological time. Relaxed clock analysis (sequences with >5% divergence) calibrated with the coccolithophore fossil record. The tree on the left shows the pattern of diversification; numbers represent Bayesian posterior probabilities of key divergences only. The tree on the right shows the corresponding divergence times. Branches are colour coded: black for sequences obtained from cultured and previously described haptophytes; grey for environmental and previously unknown sequences.

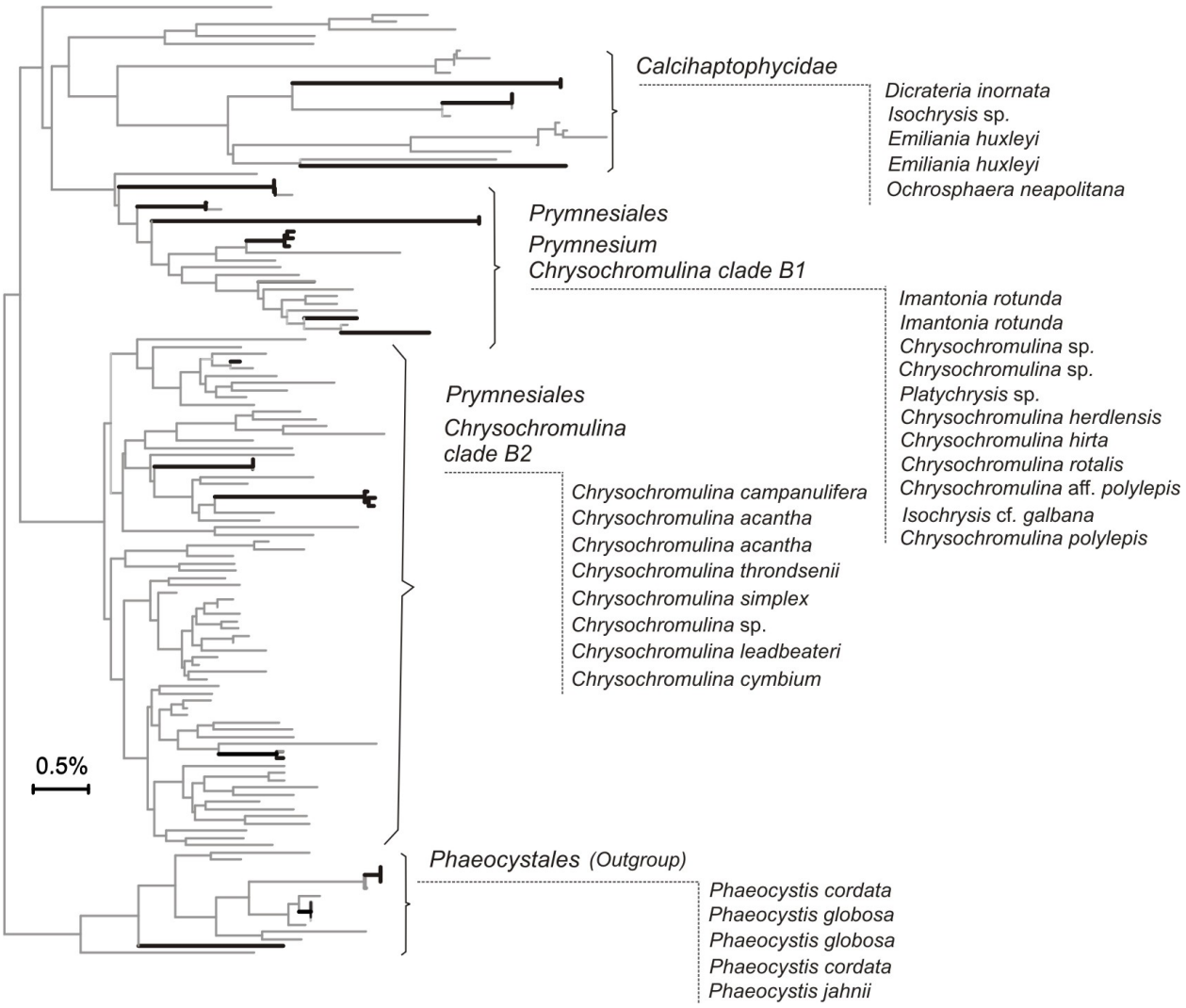


Figure S3 Chloroplastic view of eukaryotic and haptophyte diversity from the Gulf of Naples, Mediterranean sea. McDonald and collaborators[1] used chloroplastic-biased 16S rDNA primers to explore 6 environmental clone libraries over an annual cycle. 46% of the retrieved eukaryotic sequences and 73% of the total eukaryotic OTUs belonged to the Haptophyta. This overwhelming haptophyte biodiversity is reanalyzed here using the Neighbor-joining phylogenetic method based on a Tajima-Nei distance matrix. Grey branches represent the unveiled environmental diversity, integrated into taxonomically-known 16S rDNA data (black branches). Note that several taxonomic inconsistencies were removed as compared to the original dataset presented in[1].

.

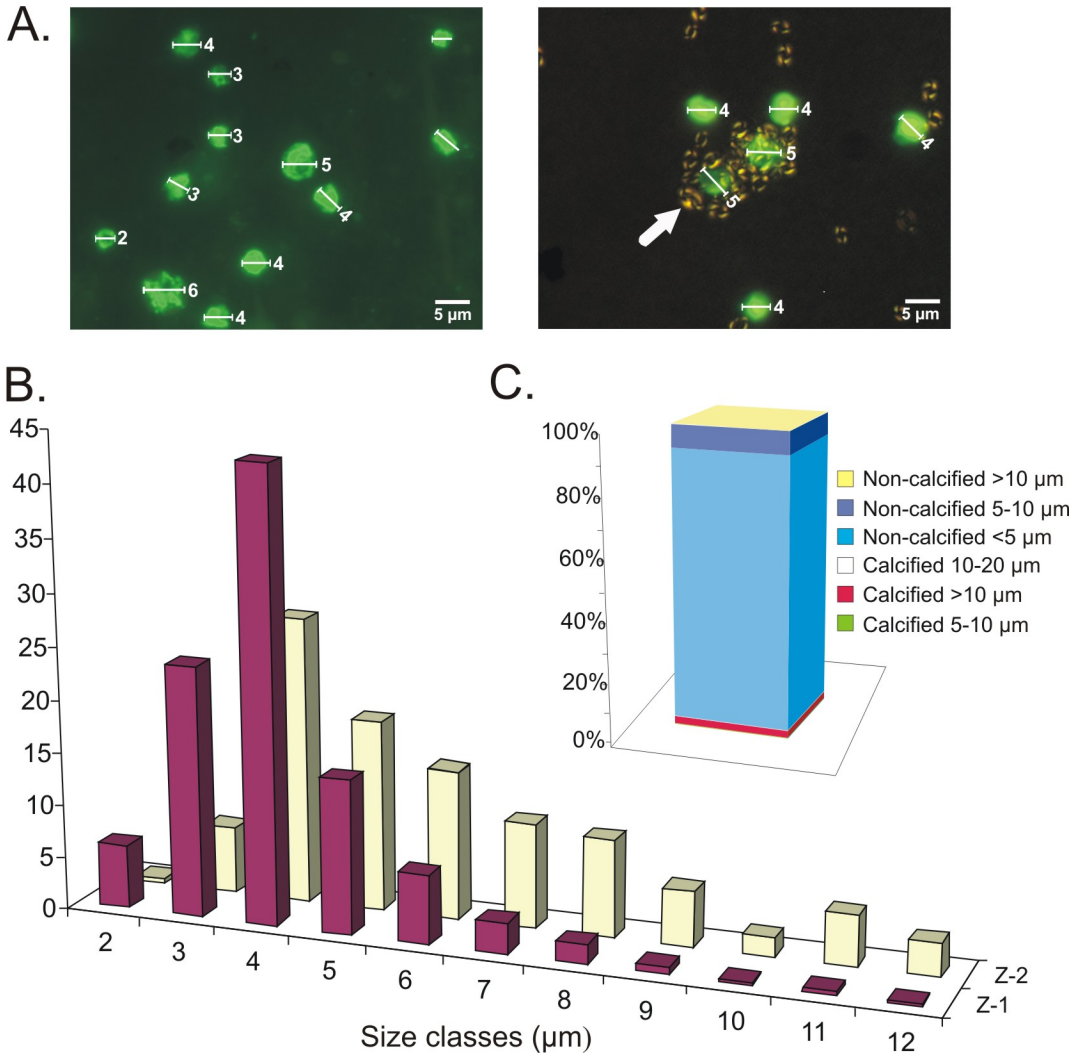


Figure S4 Abundance, size, and biovolume in non-calcifying and calcifying haptophytes. A. Haptophyte cells from 28 plankton samples from various depths in North Pacific, Mediterranean Sea, and North Atlantic waters (see Table S4) were identified by COD-FISH ($CaCO_3$ optical detection with haptophyte-specific fluorescent in situ hybridization)[2] and cell diameters were measured from 548 individuals. The white arrow in the right panel points to a single coccolith displaying typical light polarization pattern and allowing the detection of calcifying versus non-calcifying cells. The microscopy field shown in the left panel displays 12 non-calcifying cells. **B.** Relative abundance (Z-1) and relative biovolume (Z-2, estimated as a sphere [4/3*π*r3]) of *non-calcifying* haptophytes in various size-classes. C. Relative abundance of different size fractions of non-calcifying and calcifying haptophytes.

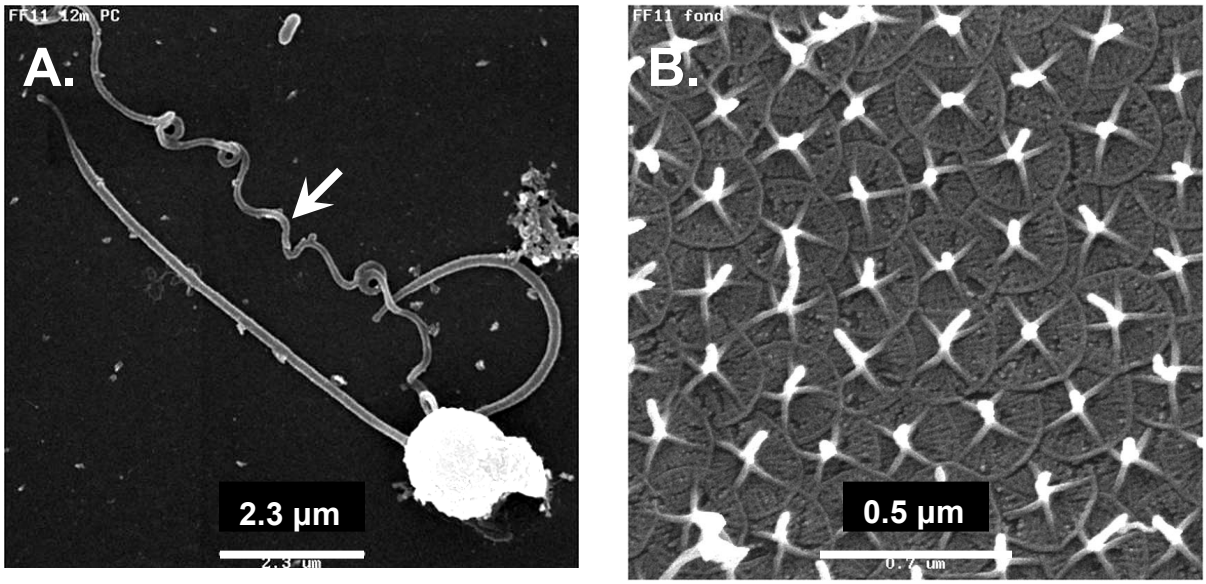


Figure S5 Tiny Chrysochromulina: haptonema and scales. A. Scanning electron microscopy image of a tiny *Chrysochromulina* sp. collected from 24m depth in the Bay of Banyuls/Mer, France, on May 15th, 2001. Such unidentified species from the genus *Chrysochromulina* are very common and diverse in oligotrophic waters, but do not seem amenable to growth in current culture media (personal communication, M-J Chrétiennot-Dinet). White arrow indicates the *haptonema*, which can be used to capture bacteria; bacterial ingestion is commonly observed in tiny haptophytes in both culture (references 25 and 26 from the core paper) and field[e.g.3,4] samples. **B.** Typical organic plate scales covering the surface of cells within the genus *Chrysochromulina* (here *C. ephippium*). *Chrysochromulina* spp., whose taxonomy is based on morphological characters, present an astonishing ultrastructural diversity of organic scales at both inter- and intra-morpho-"specific" levels[4]. Both images were graciously contributed by M-J Chrétiennot-Dinet.

Table S1: Basic features of the samples analysed in this paper. Note that seawater was pre-filtered through 3µm pore-size membrane filters and collected onto 0.2µm pore-size membrane filters.

Library name	Cruise	Station	Lat	Long	Depth (m)	Date	Vol seawater (l)	Temperature (°C)	Salinity (‰)	Fluorescence (mg/m^3)	# sequences retrieved
z11_11	ARCTIC	z11	72.5	19.6	5	8/25/2002	5	11.05	34.33	1.21	153
z61_43	ARCTIC	z61	76.32	7.98	25	8/29/2002	5	8.17	34.91	1.98	95
mv5_19	VANC 10MV	mv5	34.35	37.68	7	5/18/2003	15	20.81	35.75	0.22	144
mv5_21	VANC 10MV	mv5	34.35	37.68	85	5/18/2003	15	20.55	35.73	0.50	160
mv18_59	VANC 10MV	mv18	17.17	83.67	50	6/1/2003	15	25.03	35.04	0.24	122

Table S2: LSU rDNA total diversity estimates for each library using the Chao1 and ACE statistics. 95% confidence intervals are given in parentheses.

Sample	rDNA sequence diversity estimate			
	Unique rDNA		3% divergence cutoff	
	ACE	Chao1	ACE	Chao1
mv5_19	1412 (682 - 3076)	1099 (537 - 2401)	287 (158 - 595)	169 (114 - 288)
mv5_21	1408 (790 - 2606)	1098 (587 - 2183)	347 (210 - 622)	249 (158 - 446)
mv18_59	1084 (554 - 2230)	1147 (527 - 2664)	362 (220 - 642)	250 (154 - 463)
z11_11	756 (388 - 1585)	509 (268 - 1062)	90 (56 - 179)	88 (52 - 200)
z61_43	414 (198 - 956)	325 (157 - 769)	30 (20 - 66)	26 (19 - 62)

Table S3: Identification and origin of the haptophyte strains isolated, cultured, and characterised by electron microscopy and LSU rDNA D1-D2 sequencing, used in our study to anchor environmental genetic diversity. The strains are listed following the branching pattern of the tree in Fig. 2A (from top to bottom external black branches). All sequences except DQ980469, AF289038, and AF289040 were generated during this study.

Accession Number	Species	Culture Strain	Culture Collection	Isolation_Source
EU729452	*Platychrysis sp.*	RCC1385	Roscoff Culture Collection (RCC), France	Mediterranean - Spain
EU729451	*Platychrysis pienaarii*	RCC1392	RCC, France	unknown
EU729449	*Prymnesium sp.*	RCC1440	RCC, France	Mediterranean - Tunisia
EU729447	*Prymnesium zebrinum*	RCC1432	RCC, France	N.Atlantic - France
EU729448	*Prymnesium zebrinum*	RCC1438	RCC, France	N.Atlantic - France
EU729446	*Prymnesium sp.*	RCC1446	RCC, France	unknown
EU729445	*Prymnesium sp.*	RCC1443	RCC, France	N.Atlantic - Spain

EU729444	*Prymnesium calathiferum*	CCMP707	Center for Culture of Marine Phytoplankton (CCMP), USA	North Island, New Zealand
EU729450	*Prymnesium sp.*	RCC1450	RCC, France	Mediterranean - Tunisia
AF289038	*Prymnesium parvum*	unknown	unknown	unknown
EU729443	*Prymnesium parvum*	RCC1434	RCC, France	N.Atlantic - English Channel
EU729442	*Prymnesium sp.*	CCMP711	CCMP, USA	N.Atlantic – Maine, USA
EU729441	*Chrysochromulina sp.*	RCC1184	RCC, France	N.Atlantic - France
EU729458	*Platychrysis pigra*	RCC1390	RCC, France	Mediterranean - France
EU729457	*Imantonia rotunda*	RCC1343	RCC, France	N.Atlantic - France
EU729456	*Chrysochromulina brevifilum*	S-3	Algobank Culture Collection, France	N.Atlantic - Spain
EU729455	*Chrysochromulina ericina*	CCMP283	CCMP, USA	N.Altantic, Gulf of Maine, USA
EU729454	*Chrysochromulina hirta*	S-17	Algobank Culture Collection, France	N.Atlantic - Spain
EU729453	*Chrysochromulina cf herdlensis*	CCMP284	CCMP, USA	49.87N 142.67W
EU729440	*Chrysochromulina camella*	CCMP289	CCMP, USA	29.97N 63.86W
EU729439	*Chrysochromulina camella*	RCC1185	RCC, France	N.Atlantic - France
EU729438	*Chrysochromulina sp.*	S-14	Algobank Culture Collection, France	N.Atlantic - Spain
EU729437	*Chrysochromulina acantha*	S-6	Algobank Culture Collection, France	N.Atlantic - Spain
EU729436	*Chrysochromulina throndsenii*	S-5	Algobank Culture Collection, France	N.Atlantic - Spain
EU729435	*Chrysochromulina sp.*	No code available	RCC, France	unknown
EU729434	*Chrysochromulina simplex*	RCC1193	RCC, France	N.Atlantic - Spain
DQ980469	*Chrysochromulina sp.*	NIES 1333	NIES Collection, Japan	Pacific - Japan
EU729460	*Calcidiscus sp.*	RCC1157	RCC, France	Mediterranean - Spain
EU502878	*Calcidiscus sp.*	RCC1147	RCC, France	S.Atlantic, Namibia
EU729463	*Umbilicosphaera hulburtiana*	RCC1474	RCC, France	S.Atlantic, South Africa
EU729461	*Umbilicosphaera sibogae*	RCC1468	RCC, France	Mediterranean - Spain
EU729462	*Umbilicosphaera foliosa*	RCC1470	RCC, France	N.Atlantic - Puerto Rico
EU729464	*Coccolithus braarudii*	AC613	Algobank Culture Collection, France	N.Atlantic - English Channel
EU502875	*Jomonlithus litoralis*	RCC1354	RCC, France	Mediterranean - Spain
EU502872	*Hymenomonas globosa*	RCC1338	RCC, France	N.Atlantic - English Channel
EU729469	*Ochrosphaera neapolitana*	RCC1359	RCC, France	N.Atlantic - English Channel
EU729468	*Pleurochrysis dentata*	RCC1400	RCC, France	New Mexico - USA
EU729467	*Cruciplacolithus neohelis*	RCC1206	RCC, France	N.Atlantic - Guadeloupe
EU729466	*Calyptrosphaera*	RCC1178	RCC, France	North Sea -

	sphaeroidea			Norway
EU729465	*Helladosphaera sp.*	RCC1182	RCC, France	Pacific - Japan
EU502879	*Syracosphaera pulchra*	RCC1460	RCC, France	Mediterranean - Spain
EU729471	*Coronosphaera mediterranea*	RCC1204	RCC, France	S.Atlantic – South Africa
EU729473	*Helicosphaera carteri*	RCC1333	RCC, France	S.Atlantic – South Africa
EU729472	*Scyphosphaera apsteinii*	RCC1455	RCC, France	Mediterranean - Spain
EU729470	*Algirosphaera robusta*	RCC1128	RCC, France	Mediterranean - Spain
EU729476	*Gephyrocapsa oceanica*	RCC1289	RCC, France	Mediterranean - Spain
EU729475	*Dicrateria sp.*	RCC1207	RCC, France	Mediterranean - Morocco
EU729474	*Isochrysis galbana*	RCC1348	RCC, France	N.Atlantic - Irish Sea
EU729459	*Phaeocystis cordata*	CCMP 2495	CCMP, USA	Mediterranean - Italy
AF289040	*Phaeocystis antarctica*	unknown	unknown	unknown
EU502882	*Phaeocystis sp.*	AC618	Algobank Culture Collection, France	N.Atlantic - English Channel
EU729479	*Exanthemachrysis gayraliae*	RCC1523	RCC, France	N.Atlantic - English Channel
EU729478	*Rebecca salina*	RCC1545	RCC, France	N.Atlantic - English Channel
EU729477	*Pavlova virescens*	RCC1535	RCC, France	N.Atlantic - France
EU502883	*Pavlova pinguis*	RCC1538	RCC, France	Mediterranean - France

Table S4: Time, space, and depth information for the 28 worldwide samples used to measure haptophyte cell size (Fig. S4). For each depth, water was prefiltered through a 60µm sieve, and planktonic cells were recovered onto 0.2 µm pore-size membrane filters as in[2].

Station	Location	Date	Depth (m)
Roscoff (France), SOMLIT-Astan.	48°46′N, 3°57′W	May to July 2006 & January, April, June, October 2007	Sub-surface (n=6)
Japan, Station A	40°N, 143’E	May 2006	10, 30
Japan, Station B	40°N, 145’E	May 2006	5, 20, 30, 50, 90
Japan, Station E	34°04N, 140’E	May 2006	10, 25, 40
Japan, Station F	34°26N, 139’E	May 2006	subsurface, 30
Japan, Station G	33°21N, 140’E	May 2006	subsurface, 20, 70
Villefranche sur Mer (France), SOMLIT Point B.	43°41′N, 7°19′E	September 2007	subsurface, 20, 40, 50, 70, 150, 200

Supplementary Methods

Sampling, DNA extraction and construction of LSU rDNA clone libraries

At each sampling station (Figure S1 and Table S1) 5-15L of seawater was collected in Niskin bottles and immediately pre-filtered through a 200µm nylon mesh. The water was then gently filtered by peristaltic pump through a 3µm pore-size nucleopore polycarbonate filter (Millipore) before recovery of picoplanktonic cells on a 0.2µm pore-size Sterivex filter unit (Millipore). Filters were preserved in lysis buffer (40 mM EDTA, 50 mM Tris-HCl, 0.75 M sucrose) and stored at -80°C until genomic DNA extraction was performed as in[5]. Nuclear LSU rDNA fragments of ~1000bp containing the D1-D2 domains were PCR amplified using the forward Haptophyta-specific primer *Hapto_4* (5'-atggcgaatgaagcgggc-3'), and the reverse general eukaryote primer *Euk_34r* (5'-gcatcgccagttctgcttacc-3'). PCR reactions (98°C for 30s, 50°C for 30s, and 72°C for 60s, with initial denaturation and final extension steps) were performed over a maximum of 30 cycles to limit formation of chimeric sequences[6] using *Phusion* high-fidelity PCR DNA Polymerase (New England BioLabs) which is specifically suited for amplification of GC-rich DNA. PCR products were purified using the MinElute gel extraction kit (Qiagen) and 3'-A-overhangs were bound to DNA fragments by adding 0.2mM dATP, 1 unit of Taq DNA polymerase and 1X Taq DNA polymerase buffer to the purified PCR product and incubating for 20 min at 72°C. Classical TA-cloning into OneShot DH5α-T1 competent bacteria using the TOPO TA kit (Invitrogen) was then performed according to the manufacturer's instructions. Clone libraries were checked by PCR using the M13 forward and reverse primers and sequencing of ~25-35 random clones in both directions. The entire process of library construction was repeated until >85% of white colonies yielded high-quality sequences. Libraries were then sent to *High-Throughput Sequencing Solutions* (www.htseq.org) for random automatic picking of 200 clones, plasmid minipreps, and automatic sequencing of both strands of 150 to 200 LSU rDNA fragments per library.

Phylogenetic, molecular clock, and biogeographic analyses

Unambiguous sequences were first screened for chimeras using Check-Chimera[7], then double-checked by thorough visual inspection of all sequences producing abnormally long branches in Neighbour-Joining trees[8]. This conservative approach led to the removal

of ~13% of putative chimeric sequences from subsequent analyses. The remaining 674 environmental sequences were added to 52 nuclear LSU rDNA sequences obtained from taxonomically identified clonal haptophyte cultures from the Roscoff Culture Collection (http://www.sb-roscoff.fr/Phyto/RCC), and 3 sequences from GenBank. LSU rDNA sequences were aligned using Muscle[9] and the resulting alignment was manually inspected in *Genetic Data Environmen*t (GDE) 2.2[10]. The Akaike Information Criterion[11] was used to select the most appropriate model of nucleotide substitution: the general time-reversible model plus Γ_4 and invariable sites[12,13,14]. For each of the libraries, PAUP* 4.0b10[15] was used to build pairwise maximum likelihood distance matrices for estimation of rarefaction curves and rDNA richness based on the average neighbour algorithm implemented in DOTUR[16]. This divergence level was checked against a phylogeny of culture sequences reconstructed with MrBayes v3.1.2[17] (two independent samplers, 10^7 steps, tempering with three heated chains, burn-in of 10^4 steps). Correlations between genetic and geographic distances were assessed with Mantel tests (ecodist package in R: cran.r-project.org). A Bayesian analysis implemented in BEAST v1.4.6[18] was performed to construct the phylogeny and estimate divergence times (Figure S2). The relaxed clock analysis was calibrated with the earliest geological record for the evolution of calcification (e.g. 220 Mya[19]) and four divergence dates derived from stratigraphic data (in the order Coccolithales: the ~65 million year old first appearance of the genus *Coccolithus* and the ~24 million year old divergence between the genera *Umbilicosphaera* and *Calcidiscus*; in the order Syracosphaerales: the ~55 million year old split between the genera *Coronosphaera* and *Scyphosphaera/Helicosphaera,* and the ~25 million year old divergence between the genera *Scyphosphaera* and *Helicosphaera sensu stricto*). Minimum ages were constrained with a diffuse prior $\Gamma(1, .15)$ distribution for the onset of calcification and prior $\Gamma(1, .005)$ distributions for the fossil ages. Two data sets were analyzed: one that contained all 723 sequences and one that contained only the 184 sequences that were >5% divergent. Convergence was checked by running four independent samplers with 10^8 steps for each data set.

Assessment of the contribution of haptophytes to global oceanic photosynthetic biomass

The model in[20] was adapted to 19'-hexanoyloxyfucoxanthin (Hex), fucoxanthin (Fuco),

and zeaxanthin (Zea), the major carotenoids of haptophytes, diatoms, and photosynthetic prokaryotes, respectively. Of the >2400 worldwide HPLC-derived pigment profiles used to construct the empirical model, those sampled in stratified waters were discriminated from those located in well-mixed waters based on the ratio of the euphotic layer depth Z_{eu} (i.e., the depth at which photosynthetically available radiation is reduced to 1% of its surface value) to the mixed layer depth, Z_m. Z_{eu} was computed from the vertical profiles of Chl*a* using relevant bio-optical models[21,22], while Z_m was extracted from the Levitus global monthly-mean climatology. For stratified and mixed waters, the vertical profiles of Hex, Fuco, and Zea were sorted into "trophic categories" defined by successive intervals of Chla_{surf}. For each trophic category and each pigment, an average profile was computed. Since average pigment profiles display deterministic changes in terms of vertical shape and magnitude along the trophic gradient, they can be modelled and parameterized as a function of Chla_{surf}. Thus, a set of parameters was identified to infer vertical profiles of Hex, Fuco, and Zea for stratified or mixed water conditions, and for any given Chla_{surf} value.

The model was applied, on a pixel-by-pixel basis, to monthly composites of SeaWiFS-derived Chla_{surf} for the year 2000. First, Z_{eu} was computed from Chla_{surf} using the relationship linking Chla_{surf} to the euphotic layer-integrated Chl*a* content (Eq. 8 in[20]) and the relationship linking the euphotic layer-integrated Chl*a* content to Z_{eu}[22]. The euphotic depth was then compared to the mixed layer depth to determine whether the water column was stratified (i.e., $Z_{eu} \geq Z_m$) or mixed (i.e., $Z_{eu} < Z_m$). For stratified waters, Chla_{surf} was used to produce dimensionless profiles (with respect to depth and biomass) of Hex, Fuco, and Zea, which were then restored to physical units by multiplying depths by Z_{eu} and concentrations by the average Chl*a* concentration within the euphotic layer. For mixed waters, the surface concentration of each pigment was inferred from Chla_{surf} and extrapolated within the euphotic layer to generate uniform vertical profiles. This procedure yielded monthly depth-resolved fields of Hex, Fuco, Zea, and Chl*a* for the world ocean, which were then integrated over the euphotic zone. For each pixel, the resulting monthly integrated contents of Hex, Fuco, and Zea were converted into Chl*a* equivalents using the pigment to Chl*a* ratios determined by multiple regression analysis performed on the global pigment database[20]. The monthly Chl*a* biomasses attributed to

each group were totalled over the year to give annual mean values. These values were normalized to the annual mean euphotic layer-integrated Chl*a* content to determine the relative contribution (%) of each phytoplankton group to total phytoplankton biomass (Fig. 4). Finally, for each of the three phytoplankton groups, an annual mean Chl*a* standing stock was calculated as the sum of the annual value for each pixel multiplied by the corresponding pixel surface area. Coastal areas (bathymetry <200 m), large lakes and inland seas were not considered in this analysis.

References

1. McDonald, S.M., Sarno, D., Scanlan, D.J. & Zingone, A. Genetic diversity of eukaryotic ultraphytoplankton in the Gulf of Naples during an annual cycle. *Aquatic Microbial Ecology* **50**, 75-89 (2007).
2. Frada, M., Not, F., Probert, I. & de Vargas, C. CaCO3 optical detection with fluorescent in situ hybridization: A new method to identify and quantify calcifying microorganisms from the oceans. *Journal of Phycology* **42**, 1162-1169 (2006).
3. Nygaard, K. & Tobiesen, A. Bacterivory in Algae: A Survival Strategy During Nutrient Limitation. *Limnology and Oceanography* **38**, 273-279 (1993).
4. Eikrem, W. The class prymnesiophyceae (Haptophyta) in Scandinavian waters. Aspects of taxonomy, abundance, diversity and nutrition. PhD Thesis. Universitetet i Oslo, 200 pp (1999).
5. Diez, B., Pedros-Alio, C. & Massana, R. Study of genetic diversity of eukaryotic picoplankton in different oceanic regions by small-subunit rRNA gene cloning and sequencing. *Appl Environ Microbiol* **67**, 2932-2941 (2001).
6. Acinas, S.G., Sarma-Rupavtarm, R., Klepac-Ceraj, V. & Polz, M.F. PCR-Induced Sequence Artifacts and Bias: Insights from Comparison of Two 16S rRNA Clone Libraries Constructed from the Same Sample. *Appl. Environ. Microbiol.* **71**, 8966-8969 (2005).
7. Cole, J.R. *et al.* The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucl. Acids Res.* **31**, 442-443 (2003).
8. Hugenholtz, P. & Huber, T. Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. *Int J Syst Evol Microbiol* **53**, 289-293 (2003).
9. Edgar, R.C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**, 1792-1797 (2004).
10. Larsen, N. *et al.* The ribosomal database project. *Nucl. Acids Res.* **21**, 3021-3023 (1993).
11. Posada, D. & Crandall, K.A. Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**, 817-818 (1998).

12. Tavaré, S. Some probabilistic and statistical problems in the analysis of DNA sequences, in *DNA sequence analysis*. (ed. R.M. Miura) 57-86. (American Mathematical Society, Providence, RI 1986).
13. Hasegawa, M., Kishino, H. & Yano, T. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22**, 160-174 (1985).
14. Yang, Z. Estimating the pattern of nucleotide substitution. *Journal of Molecular Evolution* **39**, 105–111 (1994).
15. Swofford, D.L. in PAUP*: Phylogenetic analysis using parsimony (*and other methods). Version 4.0b1. (Sinauer and Associates, Sunderland, Massachusetts; 1998).
16. Schloss, P.D. & Handelsman, J. Introducing DOTUR, a Computer Program for Defining Operational Taxonomic Units and Estimating Species Richness. *Applied and Environmental Microbiology* **71**, 1501-1506 (2005).
17. Ronquist, F. & Huelsenbeck, J.P. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572-1574 (2003).
18. Drummond, A.J., Ho, S.Y.W., Phillips, M.J. & Rambaut, A. Relaxed Phylogenetics and Dating with Confidence. *PLoS Biology* **4**, e88 0699-0710 (2006).
19. Bown, P.R., Lees, J.A. & Young, J.R. Calcareous nannoplankton evolution and diversity through time, in *Coccolithophores: From Molecular Process to Global Impact*. (eds. H.R. Thierstein & J.R. Young) 481-508 (Springer, Berlin Heidelberg New York; 2004).
20. Uitz, J., Claustre, H., Morel, A. & Hooker, S.B. Vertical distribution of phytoplankton communities in open ocean: An assessment based on surface chlorophyll. *Journal of Geophysical Research* **111**, C08005 (2006).
21. Morel, A. & Berthon, J.F. Surface Pigments, Algal Biomass Profiles, and Potential Production of the Euphotic Layer: Relationships Reinvestigated in View of Remote-Sensing Applications. *Limnology and Oceanography* **34**, 1545-1562 (1989).
22. Morel, A. & S, M. Bio-optical properties of oceanic waters: A reappraisal. *Journal of Geophysical Research* **106**, 7163-7180 (2001).

ANNEX 6

Microscopy images from extant coccolithophores (Calcihaptophycidae , Haptophyta)

Miguel Frada[1,2]
Mário Cachão[2]
Sílvia Lino[5]
Ana Martins[5]
Áurea Narciso[2]
Ian Probert[1]
Jeremy Young[4]
Colomban de Vargas[1]

1. Station Biologique, Equipe EPPO-Evolution du Plancton et PaléoOcéans, Centre National de la Recherche Scientifique et Université Pierre et Marie Curie (UMR 7144), Station Biologique, 29682 Roscoff, France

2. Centro de Geologia, Faculdade de Ciências, Universidade de Lisboa, Edifício C6, Campo Grande, 1749-016 Lisboa, Portugal

4. Paleontology Department, Natural History Museum, Cromwell Road, London SW75BD, UK

5. Departamento de Oceanografia e Pescas (DOP), Universidade dos Açores

Abstract

Here we present numerous images aqcuired by light microscopy (cross-polarized and phase contrast) from extant coccolithophores species collected from various oceanic locations and from cultured strains. We hope that this collection of images will provide young students and researches interested in coccolithophore biology an accessible way to identify coccolithophore species present in natural assemblages.

Keywords

Coccolithophores, extant species, diversity, cross-polarized and phase contrast microscopy

1. Introduction

Coccolithophores include all haptophyte algae (subclass Calcihaptophycidae, (de Vargas et al. 2007)) possessing calcified scales (coccoliths) covering the cell surface (Fig.1) at least in one phase of their life cycles.

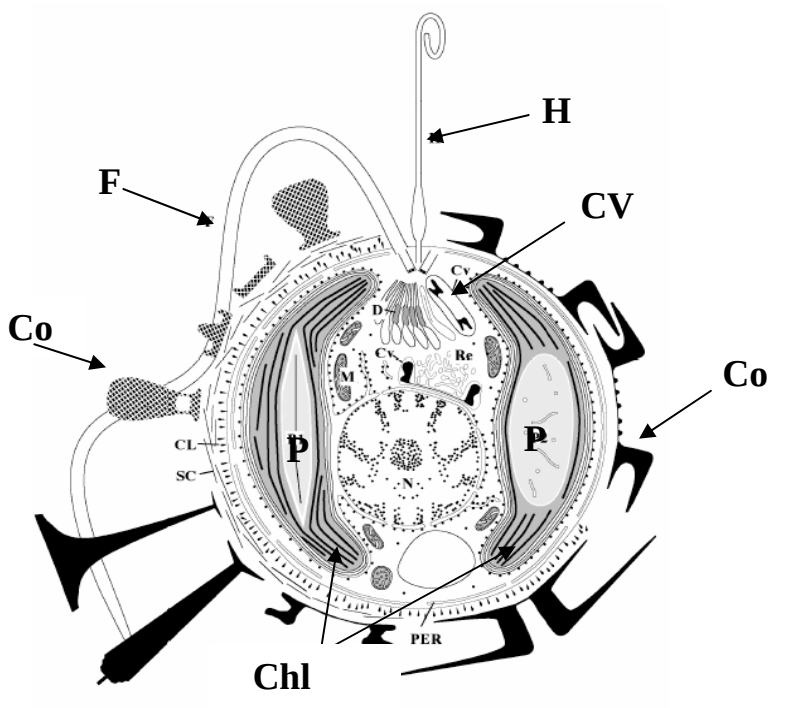


Figure 1. Schematic representation of the basic ultrastrucuture of a coccolithophore (based on (Billard & Inouye 2004). Morphological features seen in various coccolithophores are combined in a single figure: coccoliths (**Co**) covering the cell; two types of coccolith-forming vesicles (**CV**), since coccoliths are produced intra-cellularly; chloroplasts (**Chl**) with pyrenoid (**P**) typical coccolithophores; haptonema, typical of haptophytes (**H**); and flagella (**F**, haptophytes generally possess two flagella).

Indeed coccolithophore are characterized by biphasic life cycles composed of two phases morphologically distinct (heteromorphy) and possessing different ploidy levels, one beeing haplontic and the other diplontic (haplo-diplontic life cycle) (Billard & Inouye 2004, Houdan et al. 2004). This generalization is based only on limited data of chromosome counts and ploidy levels estimations made with cultures (e.g. (Gayral & Fresnel 1983, Green et al. 1996, Houdan et al. 2004)) and supplemented through the record of combination coccospheres collected in nature, which represent the transition between life phases and bearing coccoliths characteristic of both stages (e.g.(Kleijne 1991, Thomsen et al. 1991, Cros et al. 2000, Geisen et al. 2002)). From this work, it has been defined four types of life cycle among coccolithophores:

- Diploid phase bearing heterococcoliths (coccoliths composed of cycles of one or more radial arrays of elaborate and variable-shaped crysal units, (Young et al. 1992, Young et al. 2003) – haploid phase non-califying
  (known among: Noelaerhabdaceae, Hymenomonadaceae and Pleurochrysidaceae)

- Diploid phase bearing heterococcolith – haploid phase bearing holococcoliths (disc or dome-shaped coccoliths composed of numerous minute equidimensional calcite crystallites of simple shape (Young et al. 2003)
  (known among: Calcidiscaceae, Coccolithaceae, Helicosphaeraceae, Syracosphaeraceae, Rhabdosphaeraceae and Pappospheraceae)

- Phase bearing heterococcolith (possibly diploid) – phase bearing aragonitic coccoliths (possibly haploid) (Cros et al. 2000)
  (known among: Alisphaeraceae)

- Phase bearing heterococcolith (possibly diploid) – phase bearing nannoliths (possibly haploid) (Alcober & Jordan 1997)
  (known among: Ceratolithaceae)

The coccolithophores originated 225 Ma ago in Triassic (Bown 2005). Since then, they evolved and diversify, accounting today to about ~200 morphospecies (Jordan & Chamberlain 1997, Young et al. 2003), that colonize the whole ocean, constituting a major component of the planktonic communities (Okada & McIntyre 1977) (only a single freshwater coccolithophore was documented, *Hymenomonas roseola*, (Manton & Peterfi 1969). Presently, the coccolithophores represent a group of marine organism of remarkable interest to a wide range of scientists. For marine biologists and oceanographers the coccolithophores are one the main primary producers in the oceans having a distinctive role marine ecosystem functioning (Balch 2004). For biogeochemists, coccolithophores through a direct involvement in the ocean-atmosphere gas exchanges and contribution to the balast of matter into the oceans floor, play a key role in the global carbon and sulphur cycles, having therefore a major impact in the global climate regulation (Rost et al. 2003, Malin & Steinke 2004). For paleontologists and geologists, the increadibly complete and continuous

coccolithophores fossil (Bown 2005) record makes them an ideal tool for evolutionary studies and key biomarkers to perceive global changes (e.g. climate, tectonics) over the meso- and cenozoic eras.

A classical way to observe and study coccolithophores both in fresh and fossil samples is by cross-polarized microscopy. This technique is made possible because the coccoliths that cover the cells are made of calcite that is highly birefriengent and causes coccoliths to show bright extinction crosses, beeing clearly highlighted among other non-calcified marine microrganisms. Moreover, as a result of their size, composition and structure, coccoliths show ver distinct cross-polarized patterns (pseudo-extinction crosses), which allows the identification of many species or genera (for further details see ((Bown & Young 1998) and references therein).

Here we present several plates with cross-polarized and a few contrast phase microscopy pictures of coccolithophores collected from various oceanic locations. Appended to each plate we placed a scanning electronic microscopy image retrieved from the Plankton*Net website (http://planktonnet.sb-roscoff.fr), which provides further information and may allow a deeper recognition of each coccolithophore morphospecies.
Our objective is to provide the community interested in coccolithphores ecology, a guide for easy recognition and affiliation of coccolithophores from natural samples.

2. Material and Methods

The coccolithopore images presented in this work, where collected by different people and following distinct methods. In this terms we will presente the methodology according to the sample source.

Atlantic Meridional Transect 16 (May-July 2005) / KT11-06 cruise, Japan (May-June 2006) / Villefranche sur mer (September 2006)/ Hawaii HOT station (June 2005)/ Belgica cruise (May-June 2007)

All the samples from these various locations were collected both with a 5µm mesh plankton net from surface water or from water collected by a niskin bottle at different depth (for further details on AMT16 images see (Robinson et al. 2006, Poulton et al. 2007). The samples were fixed for 1h at 4ºC with paraformaldehyde (pH 8) at a final concentration of 1%. Subsequently it was filtered onto a 0.2µm anodisc filter (Whatman, Maidstone, UK) and dried at room temperature. Later, the filter was embedded in immersion oil (Olympus 04 Japan, Olympus Optical Co., Tokyo, Japan), mounted between a glass slide and a cover slip and observed in cross-polarized light and in Nomarski Interference Contrast with an Olympus

BX51 microscope (Olympus Optical Co. Ltd., Tokyo, Japan). Images were acquired with an RT-Slider Spot cooled charge coupled device digital camera (Diagnostic Instruments, Sterling Heights, MI, USA).

Azores

These samples were collected in regular bottles (May, June and July 2008), directly filtered to a nitrate cellulose membrane (0.8 µm) abd dried at room temperature. Samples preparation and observations were performed as discribed before.

Gulf of Naples

These samples were collected from surface water with a 5µm mesh size plankton net at an offshore station in the Tyrrhenian Sea (39°30' N 13°30' E) on the 24th November 2006. The sample was fixed with formaldeyde at a final concentration of 2%. Observations were made with a Zeiss Axiovert microscope equipped with a Zeiss Axiocam digital camera. For determination of the crystallographic orientation of the holococcolith crystals an aliquot of the sample was filtered onto 1µm pore size cellulose acetate filter, permanently mounted using Norland Optical Adhesive (NOA74) and examined under cross-polarised illumination using a Zeiss Axioplan photomicroscope. The methodology for this type of analysis is described in e.g. (Moshkovitz & Osmond 1989).

3. Cross-Polarized Images

Abbreviations

Cross-polarized microscopy (XpM); Phase contrast microscopy (PhM); Scanning Electronic Microscopy (SEM); The SEM images were mainly retrieved from the Plankton*Net website (http://planktonnet.sb-roscoff.fr); Heterococcolithophore [HET]; Holococcolithophore [HOL]

Images index

Plate 1. *Emiliania huxleyi*

Plate 2. *Gephyrocapsa oceanica*

Plate 3. *Reticulofenestra sessilis*

Plate 4-5. *Coccolithus braarudii*

Plate 6. *Coccolithus pelagicus*

Plate 7. *Coccolithus pelagicus azorinus* (Parente et al. 2004, Parente 2006)

Plate 8. *Calcidiscus* ssp. HET

Plate 9. *Calcidiscus quadriperforatus* HOL

Plate 10. *Oolithotus fragilis* HET

Plate 11. *Umbilicosphaera sibogae*

Plate 12. *Umbilicosphaera* ssp.

Plate 13 . *Pleurochrysis carterae*

Plate 14 . *Pleurochrysis placolithoides*

Plate 15-16. *Helicosphaera carterae*

Plate 17. *Helicosphaera wallichii*

Plate 18. *Helicosphaera hyalina*

Plate 19-22. *Scyphosphaera apsteinii*

Plate 23-24. *Scyphosphaera porosa* (Young 2008)

Plate 25. *Pontosphaera syracusana*

Plate 26. *Pontosphaera multipora*

Plate 27. *Pontosphaera japonica*

Plate 28. *Syracosphaera pulchra*

Plate 29. *Syracosphaera anthos* (Cros et al. 2000)

Plate 30. *Syracosphaera* ssp.

Plate 31. *Coronosphaera mediterranea*

Plate 32. *Michaelasarsia elegans*

Plate 33. *Michaelasarsia adriaticus*

Plate 34. *Ophiaster formosus*

Plate 35. *Calciosolenia murray*

Plate 36. *Calciosolenia brasiliensis*

Plate 37. *Discosphaera tubifera*

Plate 38. *Rhabdosphaera clavigera*

Plate 39. *Acanthoica quattrospina*

Plate 39. *Palusphaera sp.*

Plate 40. *Algirosphaera robusta*

Plate 41. *Alisphaera* sp.

Plate 42. *Braarudosphaera bigelowii*

Plate 43. *Umbelosphaera* ssp.

Plate 44. *Florisphaera profunda*

Plate 45-47. *Ceratolithus cristatus*

Plate 48. *Gladiolithus flabellatus*

4. References

Alcober J, Jordan RW (1997) An interesting association between *Neosphaera coccolithomorpha* and *Ceratolithus cristatus* (Haptophyta). European Journal of Phycology 32:91-93

Balch WM (2004) Re-evaluation of the physiological ecology of coccolithophores. In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Billard C, Inouye I (2004) What's new in coccolithophore biology ? In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Bown P (2005) Calcareous nannoplankton evolution: a tale of two oceans. Micropaleontology 51:299-308

Bown P, Young J (1998) Calcareous nannofossil biostratigraphy, Vol. Kluwer Academic Publishers, Cambridge

Cros L, Kleijne A, Zeltner A, Billard C, Young JR (2000) New examples of holococcolith-heterococcolith combination coccospheres and their implications for coccolithophorid biology. Marine Micropaleontology 39:1-34

de Vargas C, Aubry M-P, Probert I, Young J (2007) Origin and Evolution of Coccolithophores: From Coastal Hunters to Oceanic Farmers. In: Falkowski P, Knoll AH (eds) Evolution of Aquatic Photoautotrophs. Elsevier Academic Press, New York

Gayral P, Fresnel J (1983) Description, sexualité et cycle de développement d'une nouvelle Coccolithophoracée (Prymnesiophyceae): *Pleurochrysis pseudoroscoffensis* sp. nov. . Protistologica 19:245-261

Geisen M, Billard C, A.T.C. B, Cros L, Probert I, Young J (2002) Life cycle associations involving pairs of holococcolithophorids species: Intraspecific variation or cryptic speciation? European Journal of Phycology 37:531-550

Green JC, Course PA, Tarran GA (1996) The life-cycle of *Emiliania huxleyi*: A brief review and a study of relative ploidy levels analysed by flow cytometry. J Mar Syst 9:33-44

Houdan A, Billard C, Marie D, Not F, Saez A, Young G, Probert I (2004) Holococcolithophores-heterococcolithophores (Haptophyta) life cycles: flow cytometry analysis of relative ploidy levels. Systematics and Biodiversity 1:453-465

Jordan RW, Chamberlain AHL (1997) Biodiversity among haptophyte algae. Biodiversity and Conservation 6:131-152

Kleijne A (1991) Holococcolithophorids from the Indian Ocean, Res Sea, Mediterranean Sea and North Atlantic Ocean. Marine Micropaleontology 17:1-76

Malin G, Steinke M (2004) Dimethyl Sulfide Production: What is the Contribution of the Coccolithophores? In: Thierstein HR, Young JR (eds) Coccolithophores: From the molecular processes to global impact. Springer Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo

Manton I, Peterfi LS (1969) Observations on the fine structure of coccoliths, scales and the protoplast of a freshwater coccolithophorid, *Hymenomonas roseola* Stein, with supplementary observtions on the protoplast of *Cricosphaera carterae*. Proceedings Royal Society 172:1-15

Moshkovitz S, Osmond K (1989) The optical properties and crystallography of Arkhangelskiellaceae and some other calcareous nannofossils in the Late Cretaceous. In: Crux J, Heck S (eds) Nannofossils and their applications. Ellis Horwood, Chichester, p 76-97

Okada H, McIntyre A (1977) Modern coccolithophores of the Pacific and North Atlantic Oceans. Micropaleontology 23:1-55

Parente Á (2006) Morfometria Aplicada ao Cocolitóforo *Coccolithus pelagicus* s.l. à escala do Atlântico Norte, para os últimos 6 M.a., Universidade de Lisboa

Parente Á, Cachão M, Baumann K-H, Abreu L, Ferreira J (2004) Morphometry of *Coccolithus pelagicus* s.l. (Coccolithophore, Haptophyta) from offshore Portugal, during the last 200 Kyr. Marine Micropaleontology 50:107-120

Poulton AJ, Adey TR, Balch WM, Holligan PM (2007) Relating coccolithophore calcification rates to phytoplankton community dynamics: Regional differences and implications for carbon export. Deep Sea Research Part II: Topical Studies in Oceanography 54:538-557

Robinson C, Poulton AJ, Holligan PM, Baker AR, Forster G, Gist N, Jickells TD, Malin G, Upstill-Goddard R, Williams RG, Woodward EMS, Zubkov MV (2006) The Atlantic Meridional Transect (AMT) Programme: A contextual view 1995-2005. Deep Sea Research Part II: Topical Studies in Oceanography 53:1485-1515

Rost B, Riebesell U, Burkhardt S, Sultemeyer D (2003) Carbon acquisition of bloom-forming marine phytoplankton. Limnology and Oceanography 48:55-67

Thomsen HA, Østergaard JB, Hansen LE (1991) Heteromorphic life histories in artic coccolithophorids (Prymnesiophyceae). Journal of Phycology 27:634-642

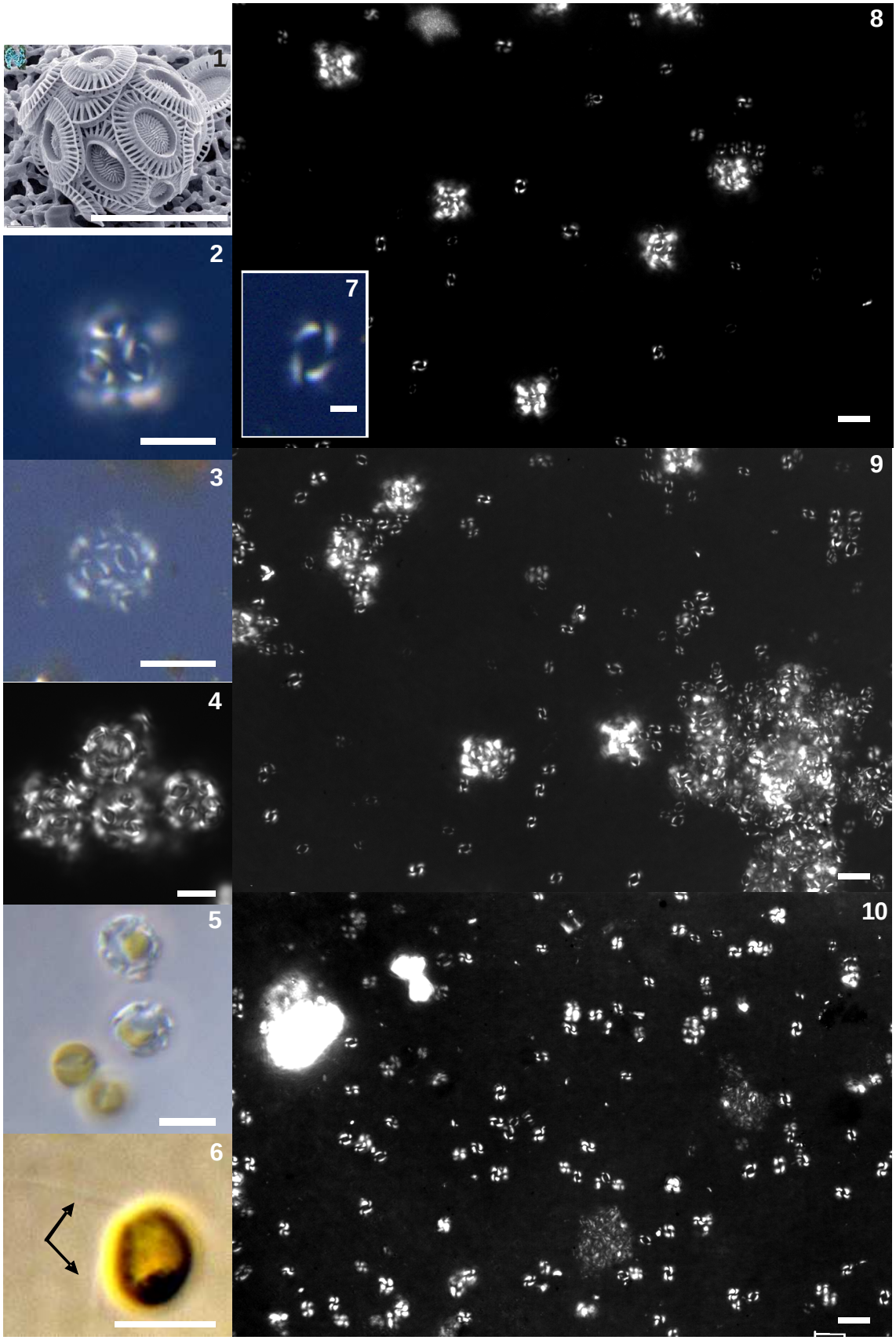
Young J, Geisen M, Cros L, Kleijne A, Sprengel C, Probert I, Østergaard J (2003) A Guide to Extant Coccolithophore Taxonomy. Journal of Nannoplankton Research

Young JR (2008) *Scyphosphaera porosa* Kamptner, 1967 Rediscovered in the Plankton. Journal of Nannoplankton Research 30:35-38

Young JR, Didymus JM, Bown PR, Prins B, Mann S (1992) Crystal assembly and phylogenetic evolution in heterococcoliths. Nature 356:516-518

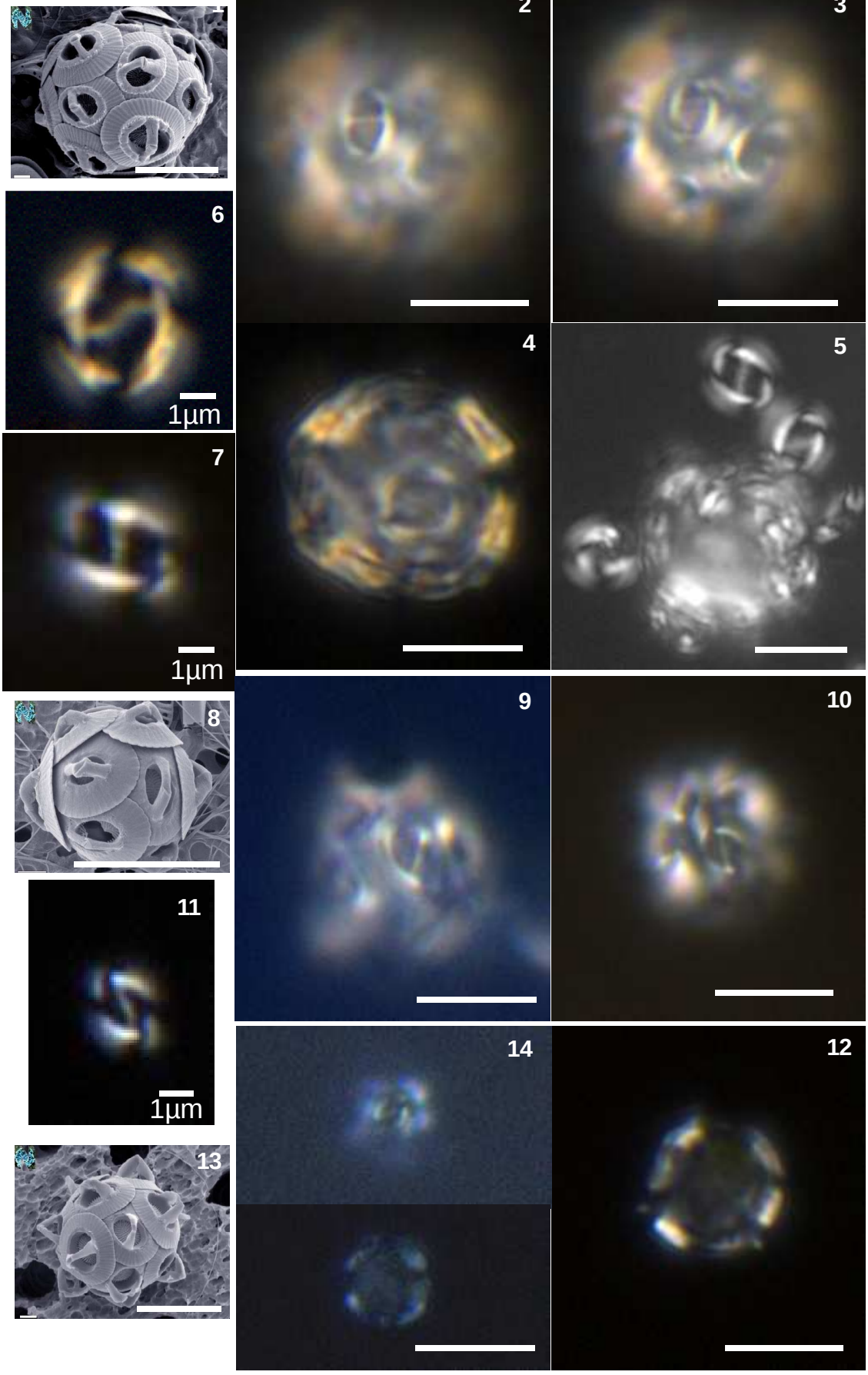
Noelaerhabdaceae

Plate 1. *Emiliania* Hay & Mohler in Hay et al. 1967
Scale bars = 5µm (exception : figure 7b = 1µm)
1. *Emiliania huxleyi* (Lohmann 1902) Hay & Mohler in Hay et al. 1967, diploid phase [HET] (Plankton*Net; Authors: Claudia Sprengel and Jeremy Young; Copyright: Jeremy Young , NHM); SEM.
2-4. *E huxleyi* diploid phase [HET]; 04º S - 25º W South Atlantic (surface) [AMT16 cruise, June 2005], 30º32' N - 28'33'W Azores , Faial (30m) [May 2008] and 40°N - 145'E Japan (surface water) [Tansei Maru KT11-06 cruise, May 2006] respectively; XPL.
5. *E huxleyi* diploid phase [HET]; culture: RCC 1249; PhM
6. *E huxleyi* haploid biflagellated (arrow) phase; culture: RCC 1249; PhM.
7. *E huxleyi* detached coccolith; XPL.
8-10. Series of images from different phases of a *E huxleyi* diploid phase bloom in the english channel [Belgica cruise, May 2007]: 8) Exponentially growing phase – healthy cells, and few detached coccoliths; 9) Stationary phase – increased amount of detached coccoliths, appearence of clumps of cellular debries; 10) termination of the bloom- cellular debries and detached lith, very few cells are present; XPL.


1
2
3
4
5
6
7
8
9
10

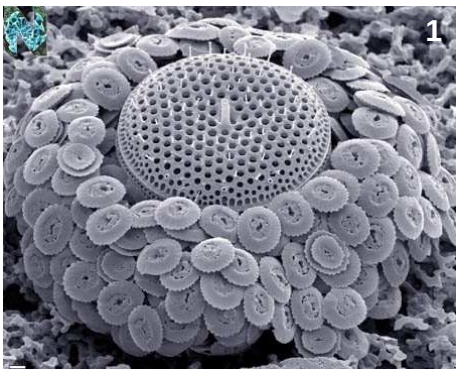
Noelaerhabdaceae

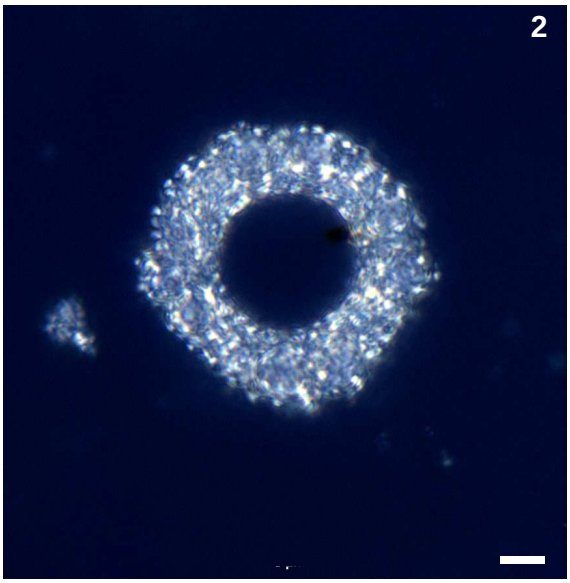
Plate 2. *Gephyrocapsa* Kampter 1943
Scale bars = 5µm (exception : = 1µm)
1. *G oceanica* Kampter 1943, diploid phase [HET] (Author: Markus Geisen; Copyright: Jeremy Young, The Natural History Museum, London; SEM.
2-4. *G oceanica*, diploid phase [HET] (surface water); 30º32' N - 28'33'W Azores , Faial (30m) [May 2008]; XPL
5. *G oceanica*, diploid phase [HET] (surface water); 34°26N - 139'E Japan [Tansei Maru cruise, May 2006]
6-7. *G oceanica* detached coccoliths; XPL
8. *G mullerae* Bréhéret 1978, diploid phase [HET] (Plankton*Net; Authors: Markus Geisen and Jeremy Young; Copyright: Jeremy Young, NHM); SEM.
9, 10 and 12. *G mullerae*; 30º32' N - 28'33'W Azores , Faial (30m) [May 2008] (surface water); XPL
11. *G mullerae* detached coccolith; XPL
13. *G ericsonii* [McIntyre & Bé 1967, diploid phase [HET] (Plankton*Net; Authors: Jeremy Young; Copyright: Jeremy Young, NHM); SEM.
14. *G ericsonii* [HET], 30º32' N - 28'33'W Azores (surface) [June 2008], July 2008; XPL

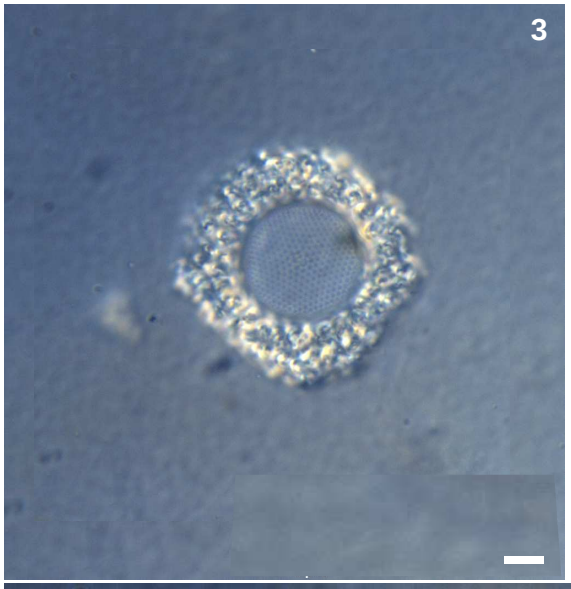

1
2
3
6
1µm
4
5
7
1µm
8
9
10
11
1µm
14
12
13

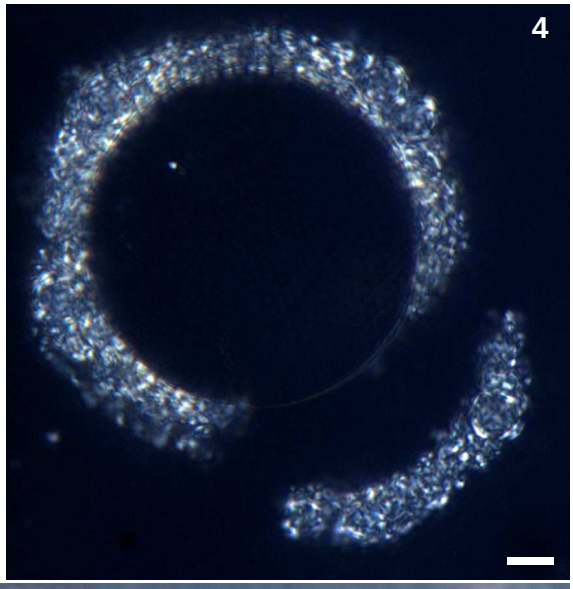
Noelaerhabdaceae

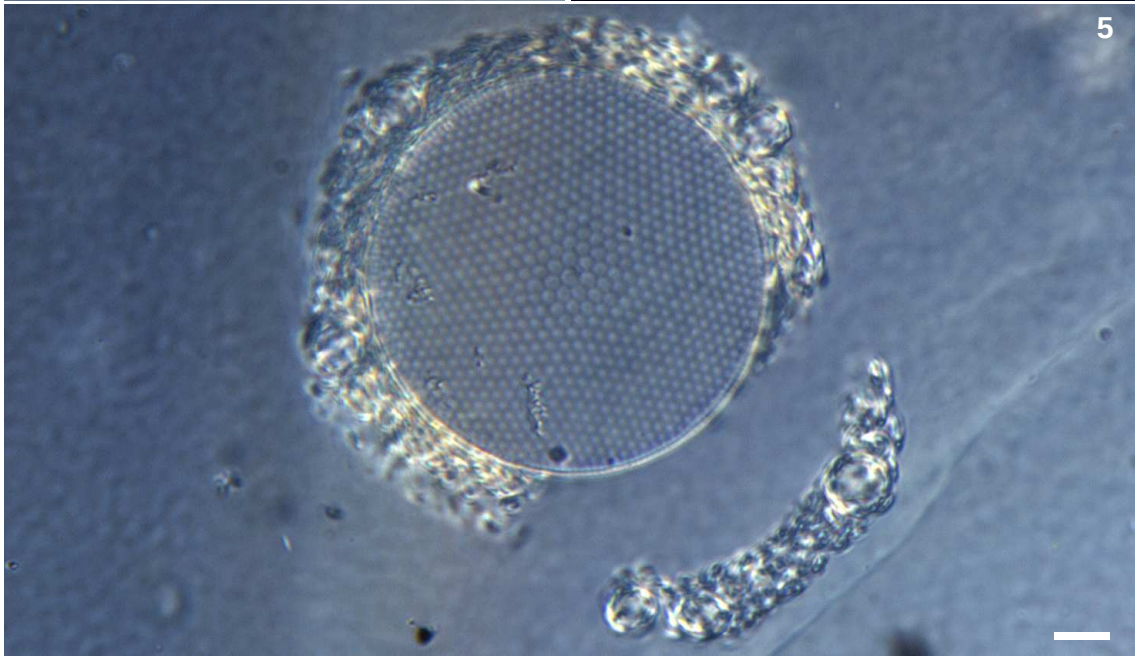
Plate 3. *Reticulofenestra sessilis* Hay, Mohler & Wade 1966
Scale bars = 5µm
1. *R. Sessilis* (Lohmann 1902) Jordan & Young 1990, cells [HET] associated with a centric diatom *Thalassiosira* (Plankton*Net; Authors: Vita Pariente and Jeremy Young; Copyright: Jeremy Young, NHM); SEM.
2-4 *R. Sessilis* cells [HET] associated with a diatom; 31º 49'S - 01º30'E South Atlantic [AMT16 cruise, May 2005] (surface water); XPL and PhM.


1


2

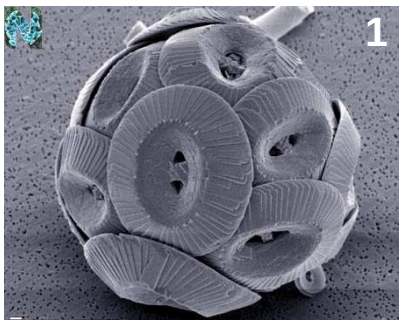

3

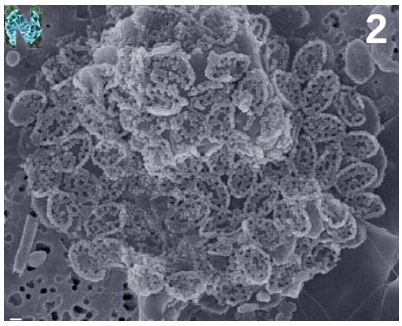

4

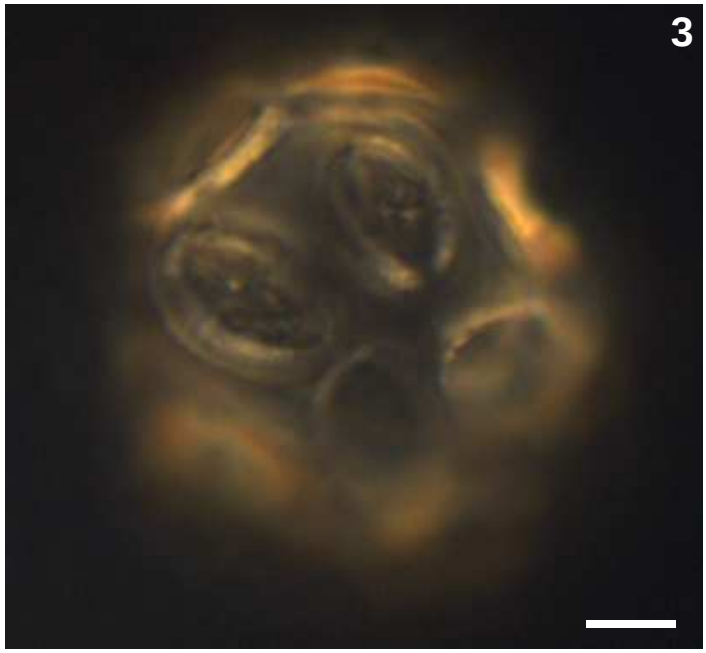

5

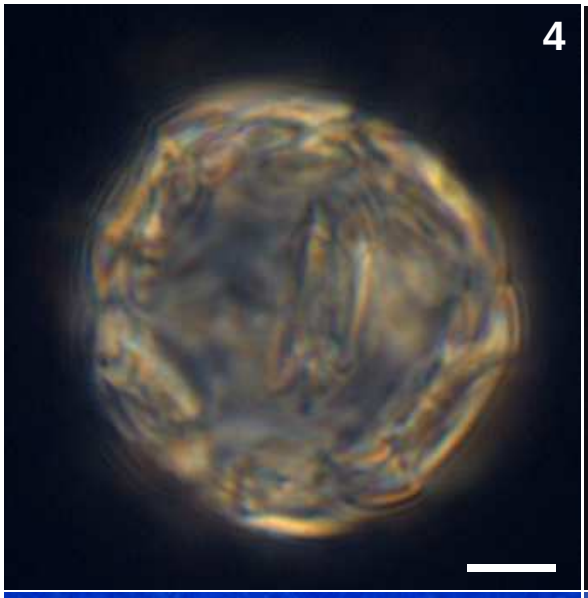
Coccolithaceae

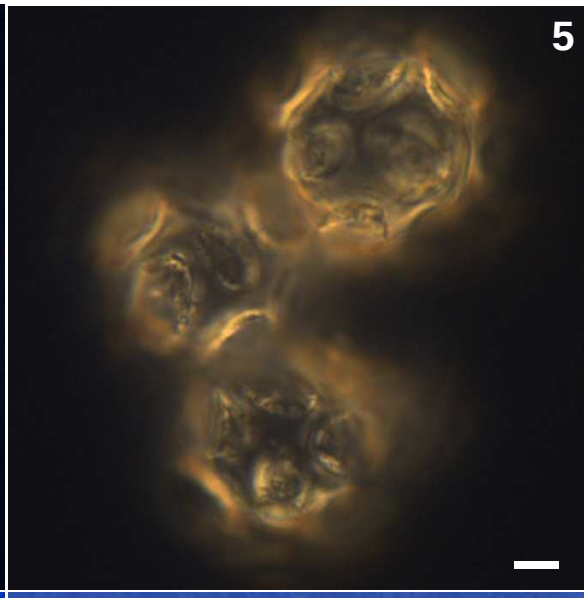
Plate 4. *Coccolithus braarudii* (Gaarder 1962) Geisen et al. 2002 [HET]
Scale bars = 5µm
1.*C. braarudii* (Plankton*Net; Authors: Richard Lampitt and Jeremy Young; Copyright: Jeremy Young, NHM); SEM
2.*C.* braarudii HOL ('Crystallolithus braarudii'); holococcolith phase (haploid) - collapsed coccosphere. (Plankton*Net; Authors: Lluisa Cros; Copyright: Lluisa Cros, CSIC-ICM Barcelona); SEM
3-5. *C. braarudii*[HET], culture strain RCC1197; XPL
6-7. *C. braarudii*[HOL], culture strain RCC1197; XPL

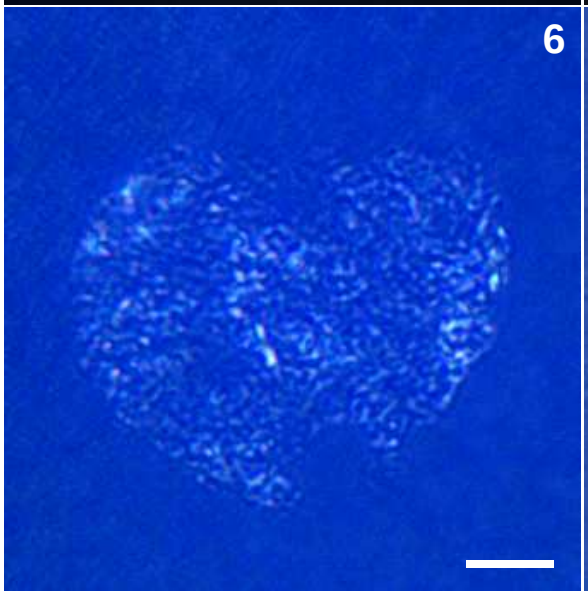

1

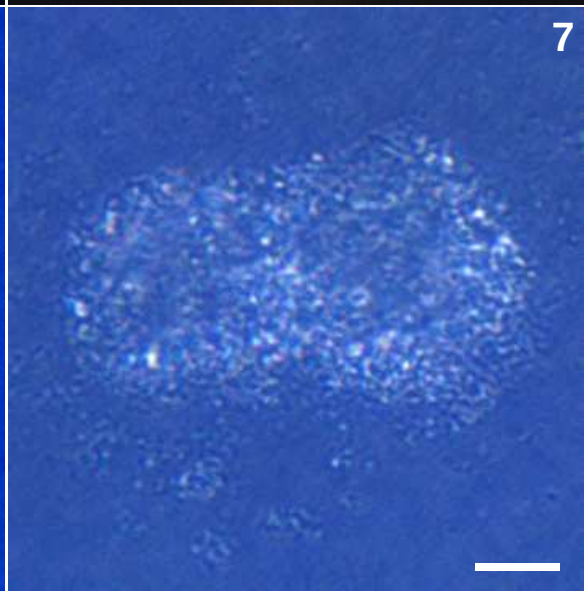

2


3


4


5

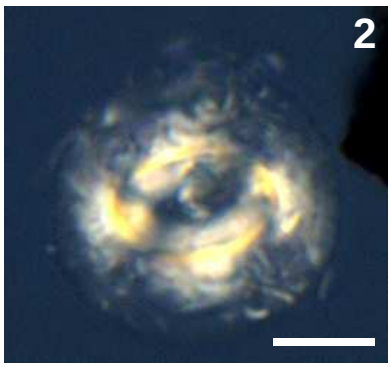

6

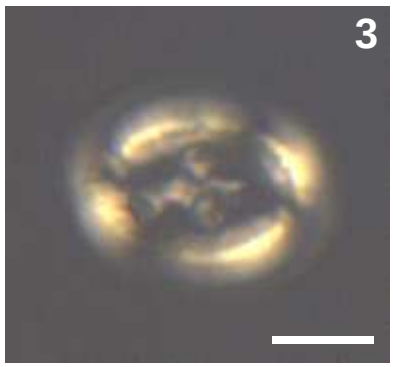

7

Coccolithaceae

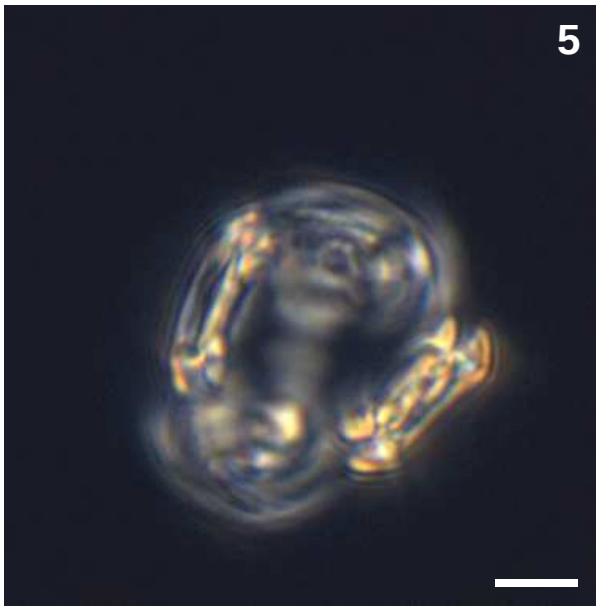
Plate 5. *Coccolithus braarudii* (Gaarder 1962) Geisen et al. 2002 [HET] Scale bars = 5µm
1-2.*C. braarudii* coccolith [HET]; XPL
3. *C. braarudii*, primary stages of coccolith formation; XPL
4-6. *C. braarudii* [HET] RCC1197, destroied coccospheres ; XPL

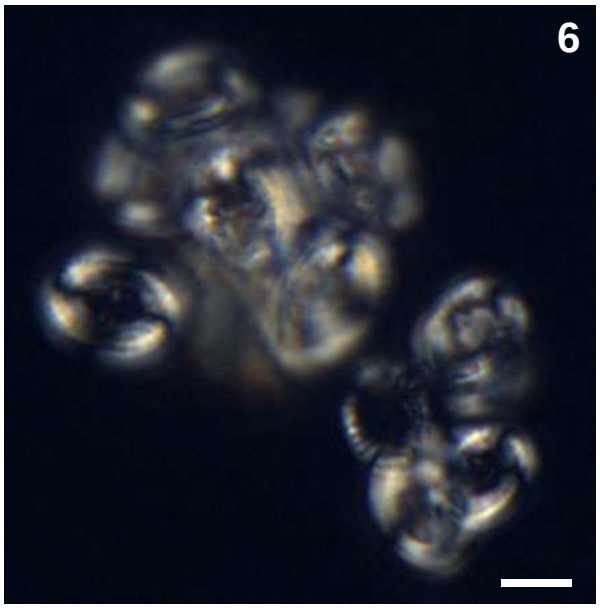

1


2


3

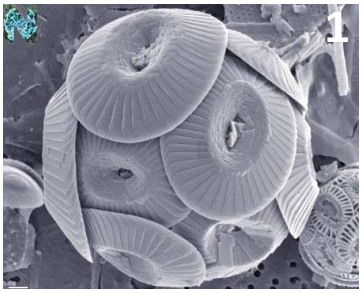

4

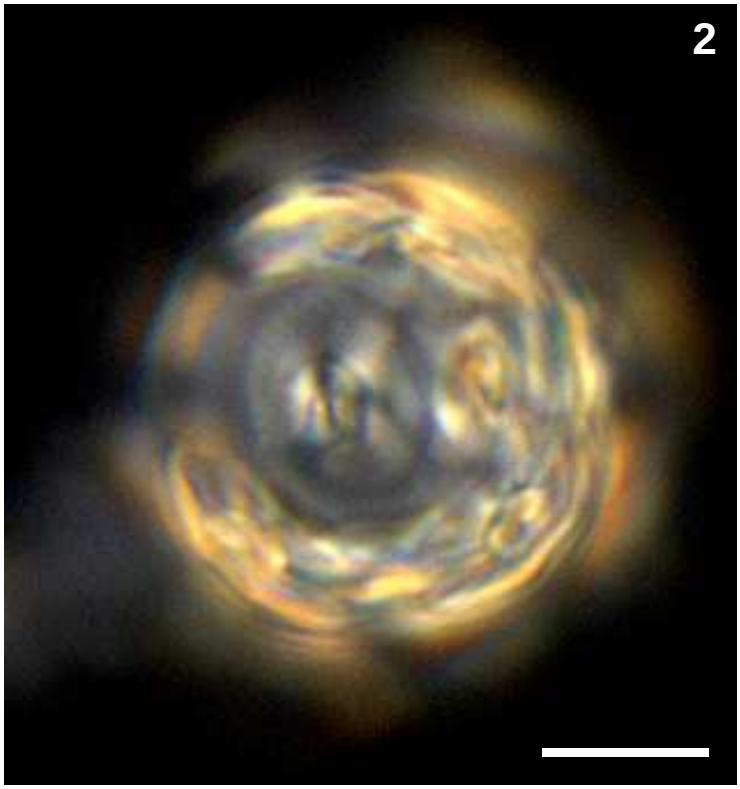

5


6

Coccolithaceae

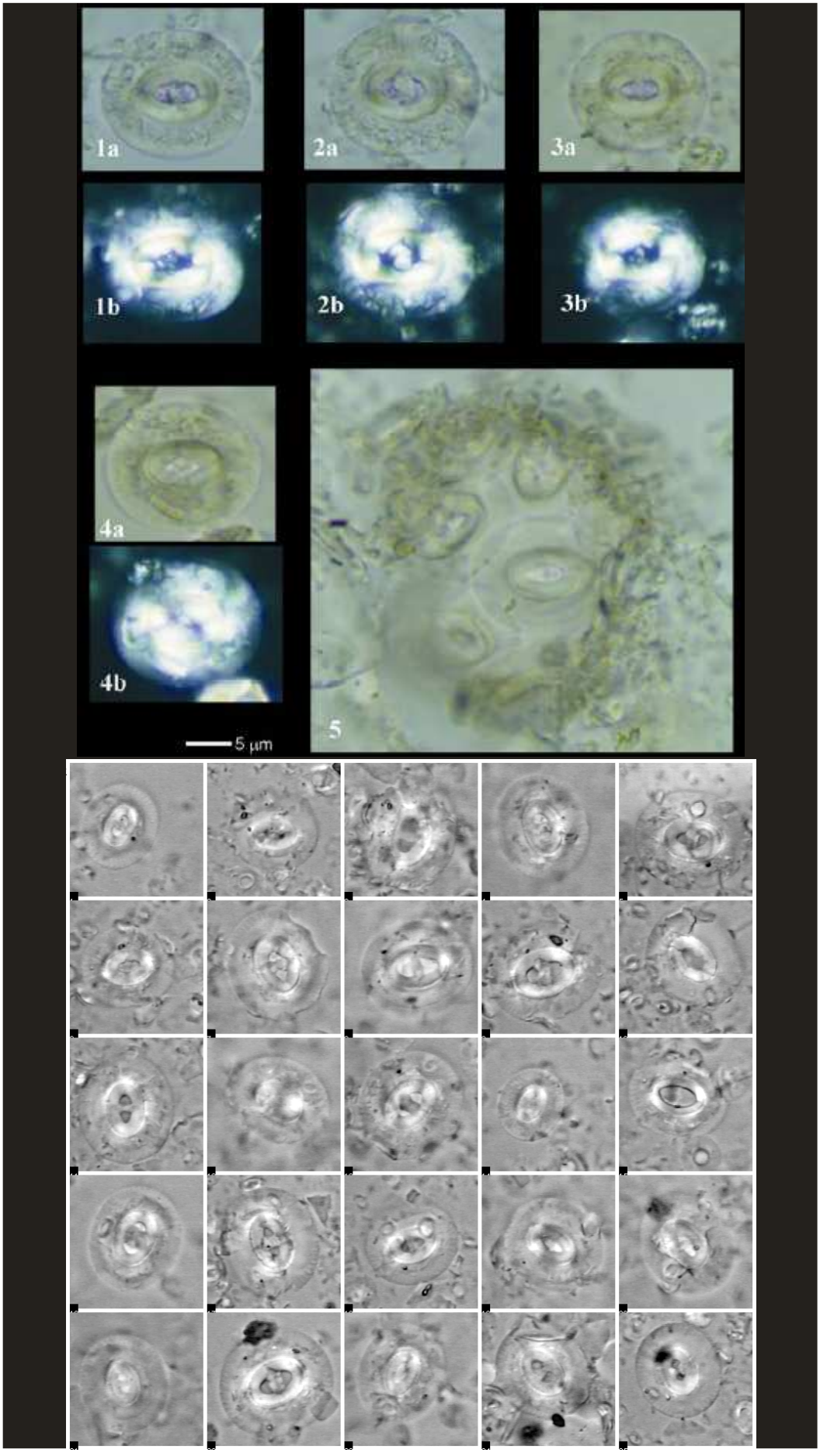
Plate 6. *Coccolithus pelagicus* (Wallich 1877) Schiller 1930 [HET]
Scale bars = 5µm
1. *C. pelagicus* [HET] (Plankton*Net; Authors: Jeremy Young; Copyright: Jeremy Young, NHM); SEM
2. *C. pelagicus*; 30º32’ N - 28’33’W Azores , Faial (30m) [May 2008] (surface water); XPL





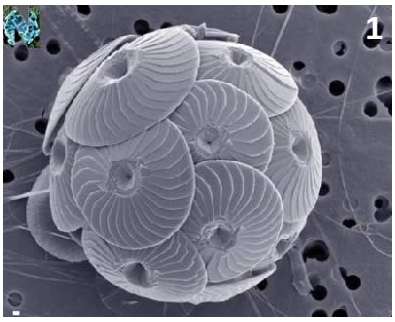
Coccolithaceae

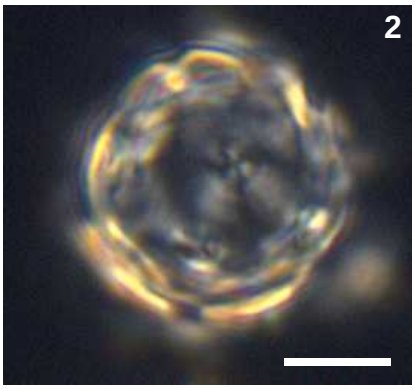
Plate 7. *Coccolithus pelagicus azorinus*
(image from Aurea Parente PhD thesis)
1b-4b. Distal view of *C. pelagicus azorinus*
(Sample collected at 1370-1371m depth from the sediment); XPL
5. *C. pelagicus azorinus* cocospheres from the same samples; XPL

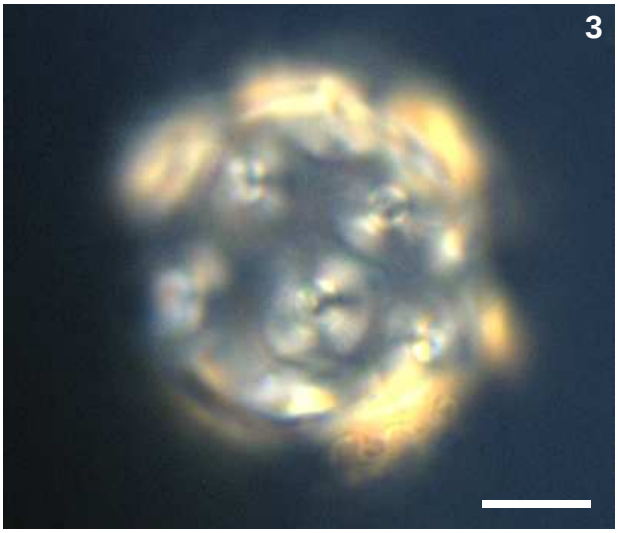

1a
2a
3a
1b
2b
3b
4a
4b
5
5 µm

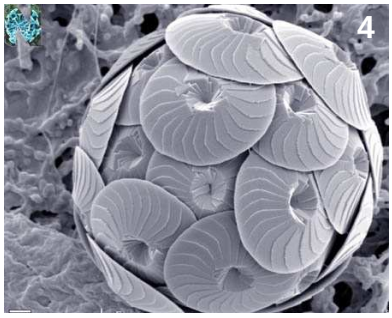
Calcidiscaceae

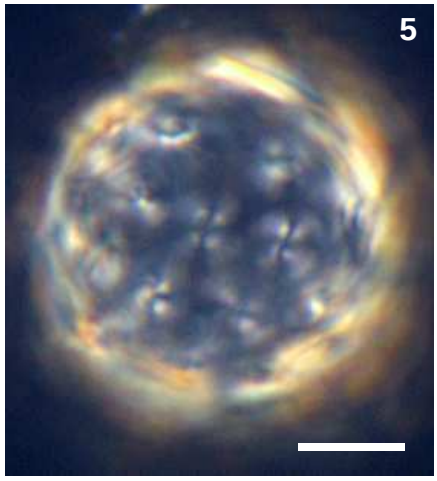
Plate 8. *Calcidiscus* Kampter 1950
Scale bars = 5µm
1. *C. quadriperforatus* [HET] (previously *C. pelagicus*- large morphotype) (Plankton*Net; Authors: Brian Pedder; Copyright: Jeremy Young, NHM); SEM
2-3. *C. quadriperforatus*;]; 01º37 S - 24º59 W (surface) South Atlantic [AMT16 cruise, June 2005]; and 26º31 S - 17º13 W (surface) South Atlantic [AMT16 cruise, May 2005]; XPL
4. 1. *C. pelagicus* [HET] -intermediate morphotype - (Plankton*Net; Authors: Markus Geisen; Copyright: Jeremy Young, NHM); SEM
5-7. *C. pelagicus* [HET] –intermediate morphotypes]; 20º11' S – 24º59' W (surface) South Atlantic [AMT16 cruise, May 2005]; XPL
8. *C. pelagicus* [HET] –small morphotype- (Plankton*Net; Authors: Markus Geisen; Copyright: Jeremy Young, NHM); SEM
9. Possibly a *C. pelagicus* [HET] – small, 30º32' N - 28'33'W Azores , Faial (30m) [July 2008] (surface water); XPL

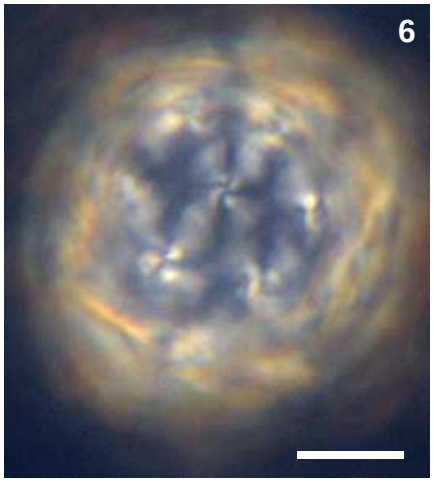

1

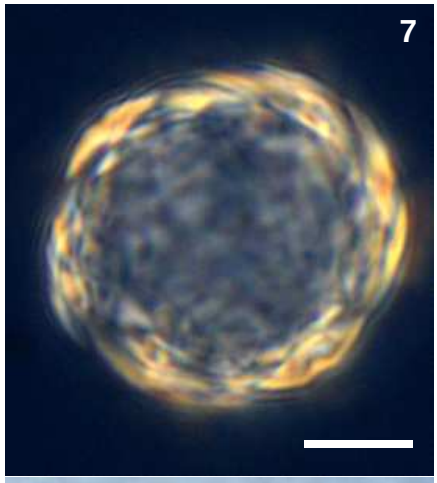

2

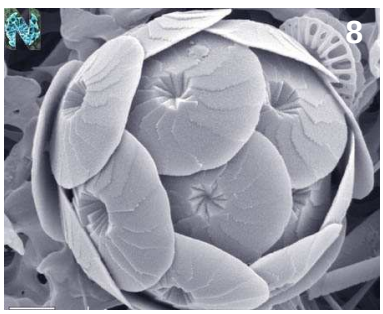

3


4


5


6

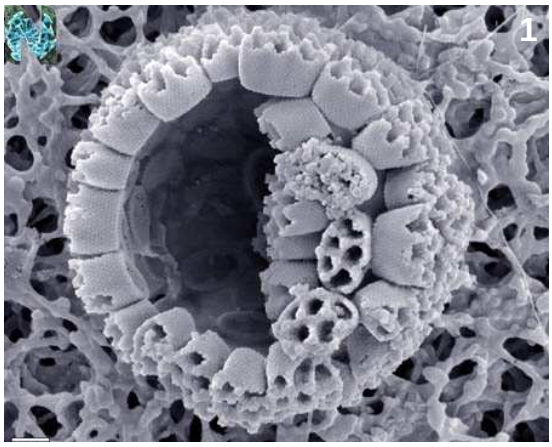

7

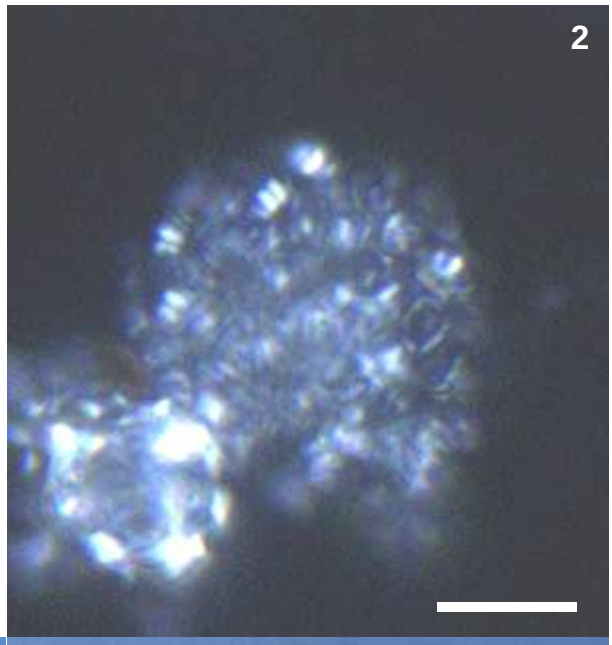

8

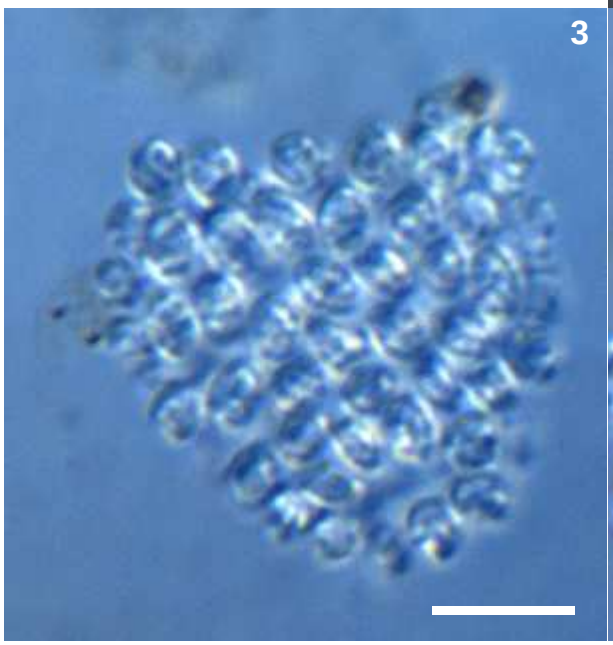

9

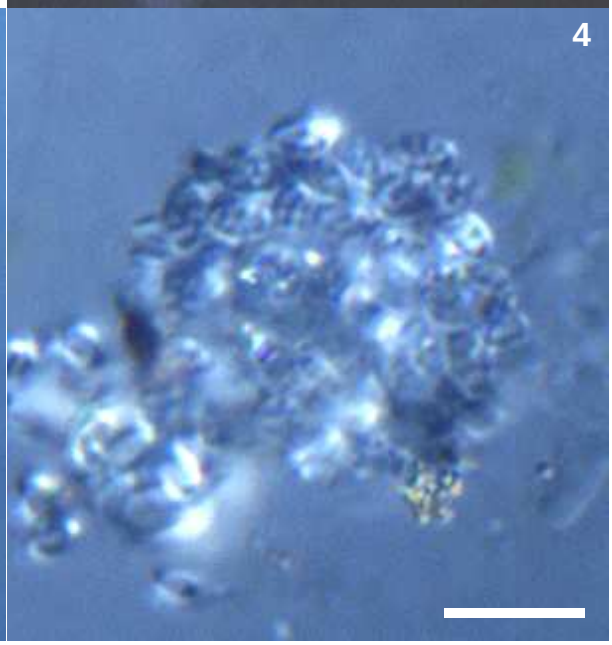
Calcidiscaceae

Plate 9. *Calcidiscus quadriperforatus* [HOL] (‘*Syracolithus quadriperforatus*’)
Scale bars = 5µm
1. *C. quadriperforatus* (Plankton*Net; Authors: Claudia Sprengel Jeremy Young; Copyright: Jeremy Young, NHM); SEM
2. *C. quadriperforatus*;]; 26º31 S - 17º13 W (surface) South Atlantic [AMT16 cruise, May 2005]; XPL and PhM


1

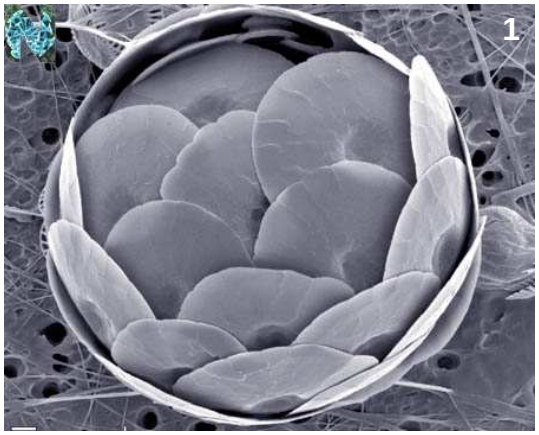

2

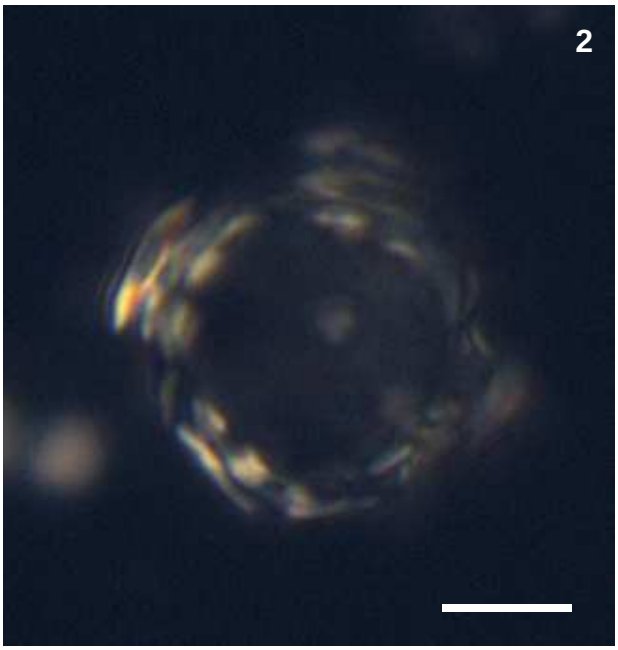

3

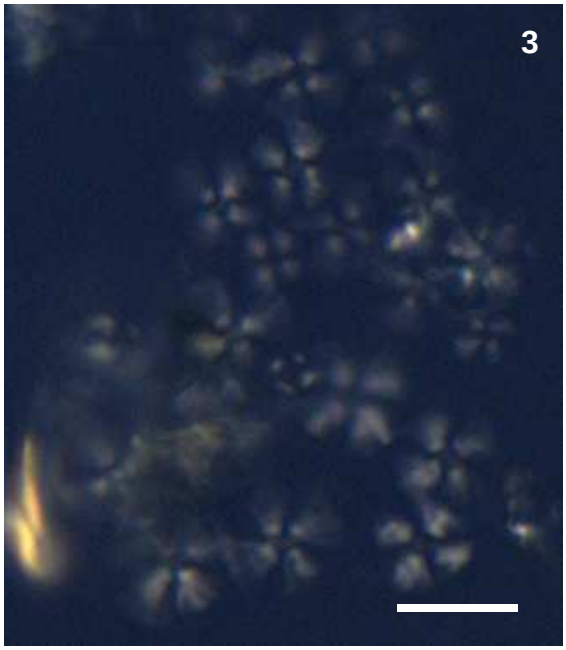

4

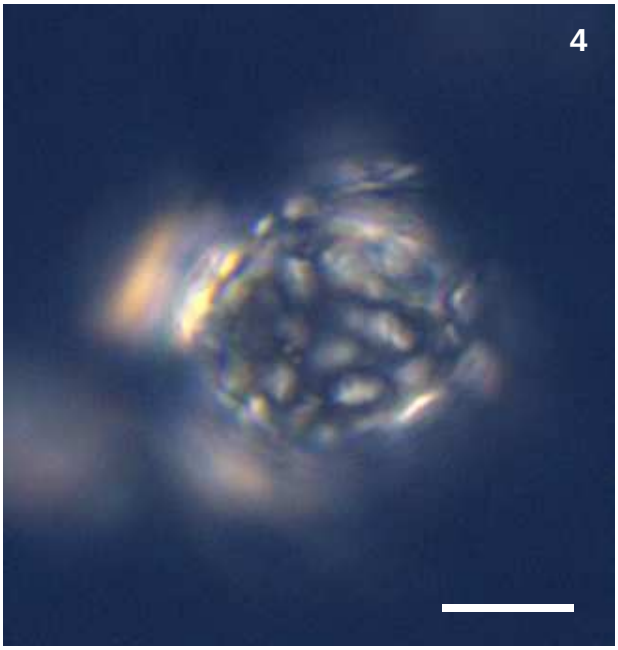
Calcidiscaceae

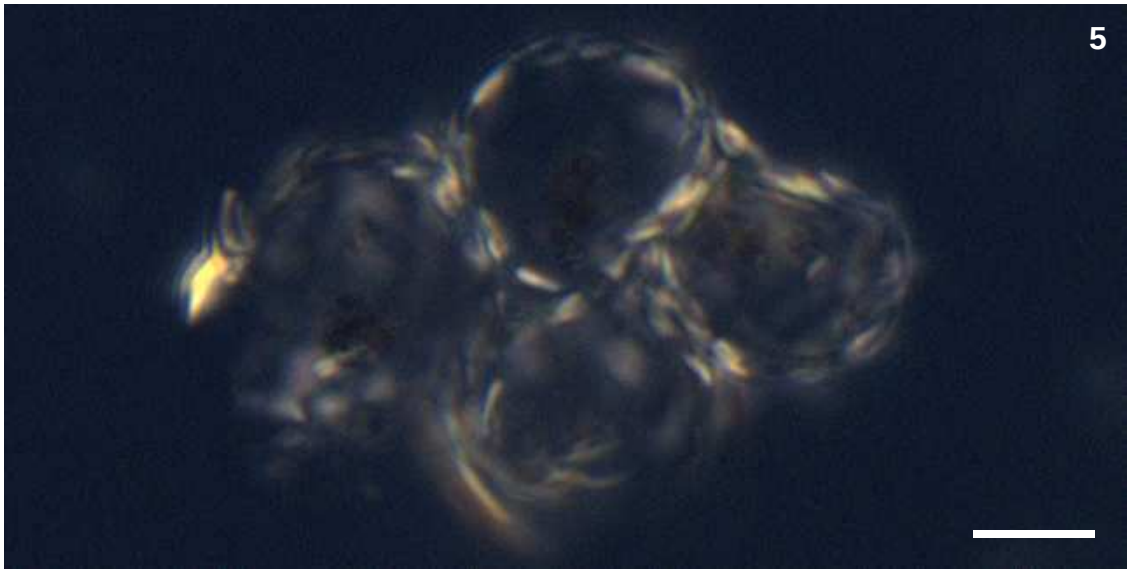
Plate 10. *Oolithotus fragilis* (Lohmann 1912) Martini and Müller 1971 [Coccolithophora]
Scale bars = 5µm
1. *O. fragilis* [HET] (Plankton*Net; Authors: Markus Geisen; Copyright: Jeremy Young, NHM); SEM
2 and 4. *O. Fragilis* [HET], culture strain RCC; XPL
3. *O. Fragilis* dispersed heterococcoliths; XPL
5. *O. Fragilis*, group of four cells [HET], culture strain RCC; XPL


1


2

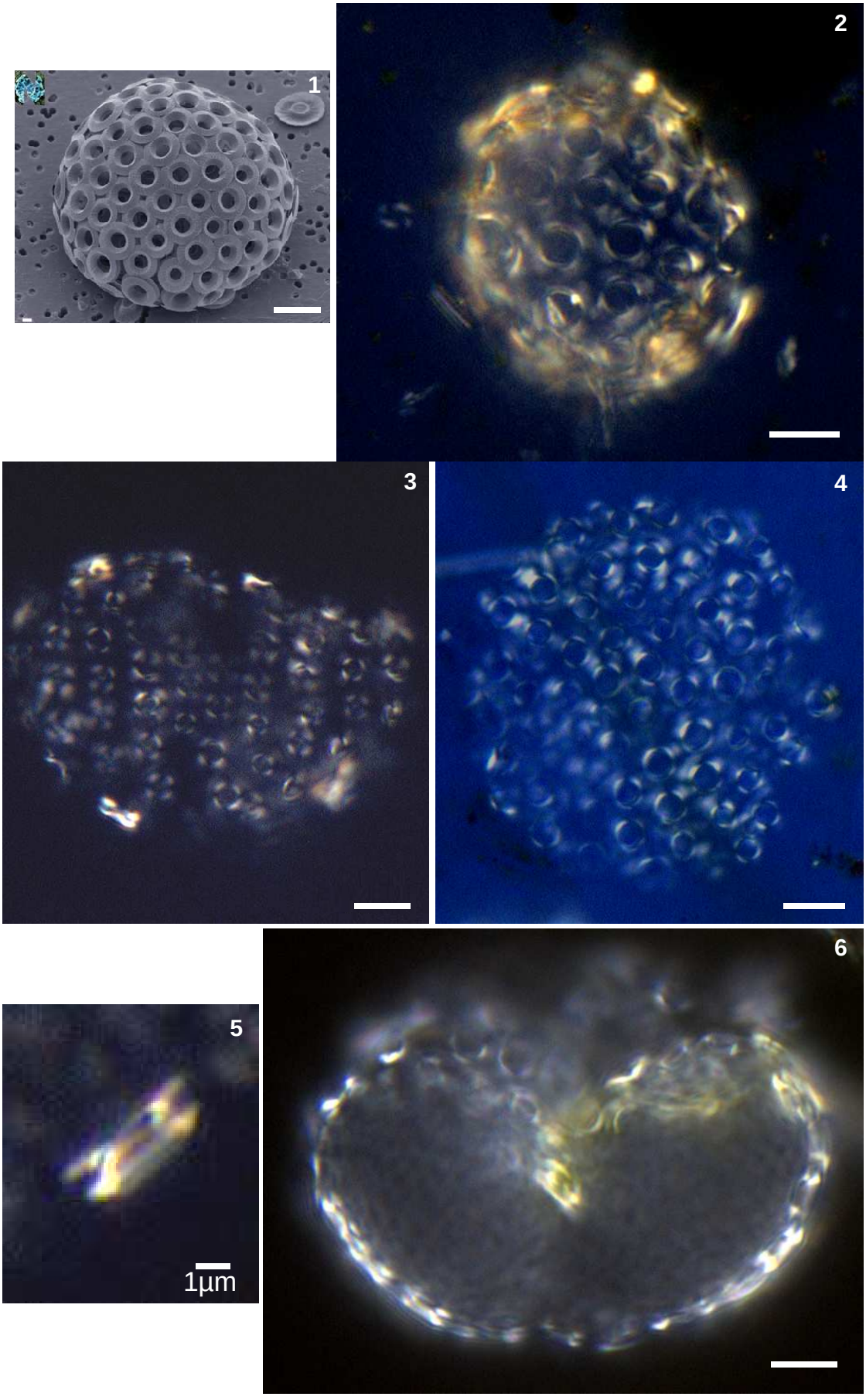

3


4


5

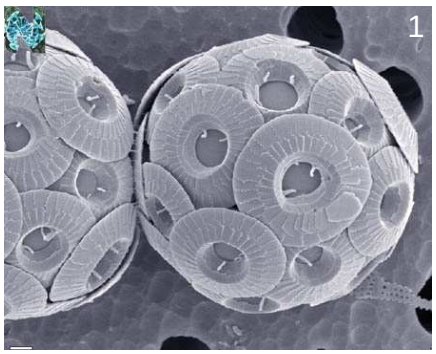
Calcidiscaceae

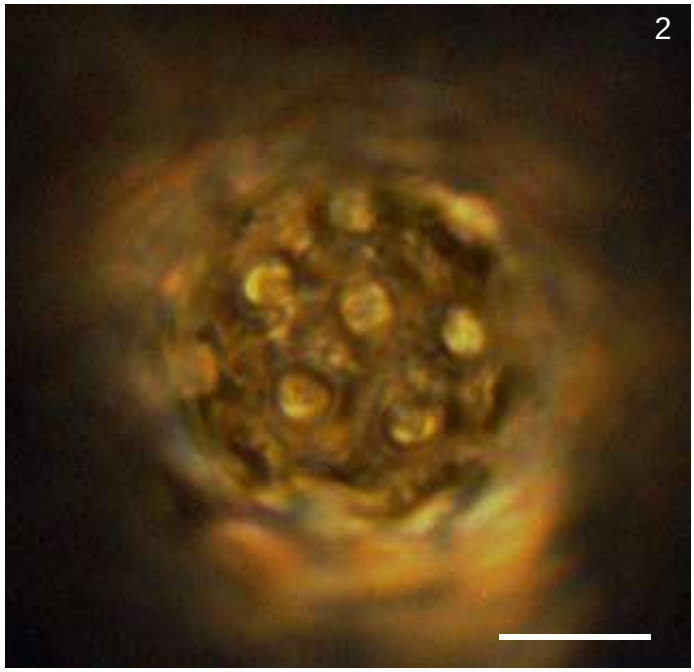
Plate 11. *Umbilicosphaera* Lohmann 1902
Scale bars = 5µm (exception : = 1µm)
1.U sibogae [HET] (Weber-van Bosse 1901) Gaarder 1970 [*Coccosphaera*] (Plankton*Net; Authors: Jeremy Young and Brian Pedder; Copyright: Jeremy Young, NHM); SEM
2-5. *U sibogae* [HET]; 01º37 S - 24º59 W, 20º11 S - 24º59 W and 04º16 N - 27º01 W South/North Atlantic (surface water) [AMT16 cruise, June 2005]; 5- detached coccolith; XPL
6. *U sibogae* [HET]; 30º32' N - 28'33'W Azores , Faial (30m) [May 2008]; XPL

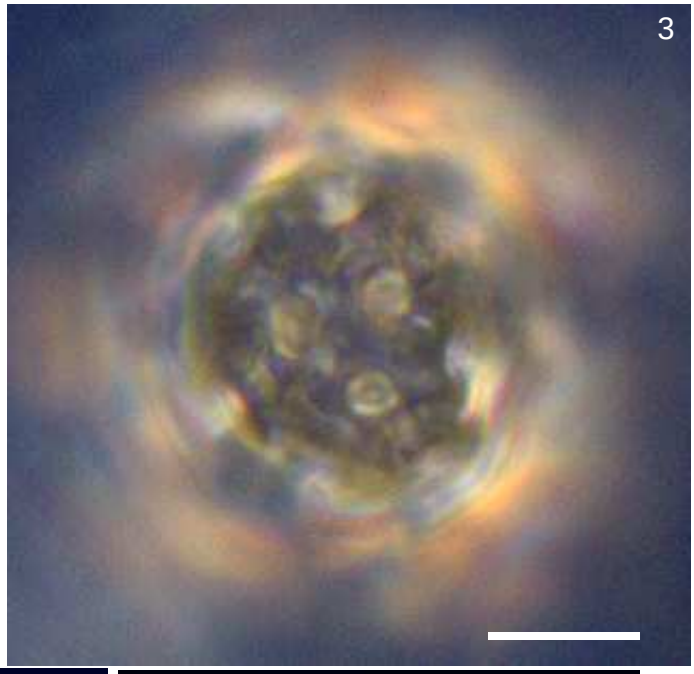


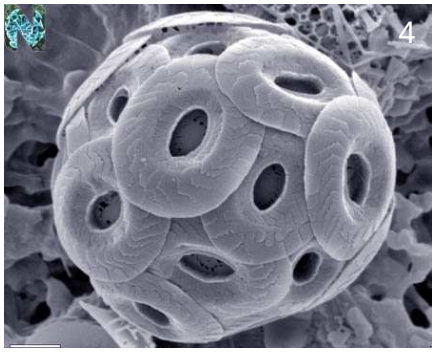
Calcidiscaceae

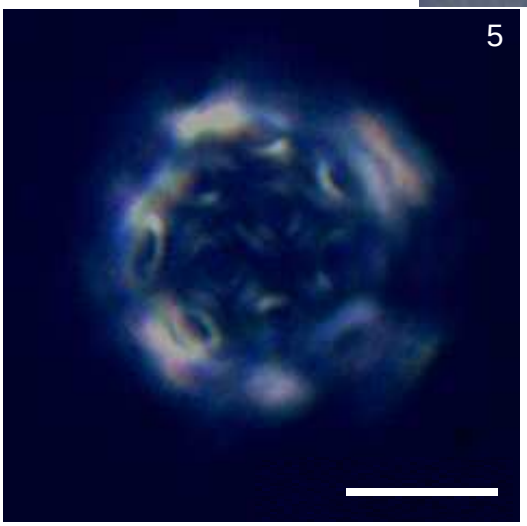
Plate 12. *Umbilicosphaera* Lohmann 1902
Scale bars = 5µm
1. *U. foliosa* [HET] (Kampter 1963, ex Kleijne 1993) Geisen in Saez et al. 2003 [*Cycloplacolithus*] (Plankton*Net; Authors: Markus Geisen; Copyright: Jeremy Young, NHM); SEM.
2-3. *U foliosa* [HET]; 26º49 S - 10º30 W (surface), 18º57 N - 34º12 W (surface) South/North Atlantic [AMT16 cruise, May/June 2005]; XPL
4. *U hulburtiana* [HET] Gaarder 1970 (Plankton*Net; Authors: Claudia Sprengel and Jeremy Young; Copyright: Jeremy Young, NHM); SEM.
5-6. *U hulburtiana* [HET]; 22º52 S - 24º59 W (surface), 01º37 S - 24º59 W (70m) South Atlantic [AMT16 cruise, May/June 2005]; XPL

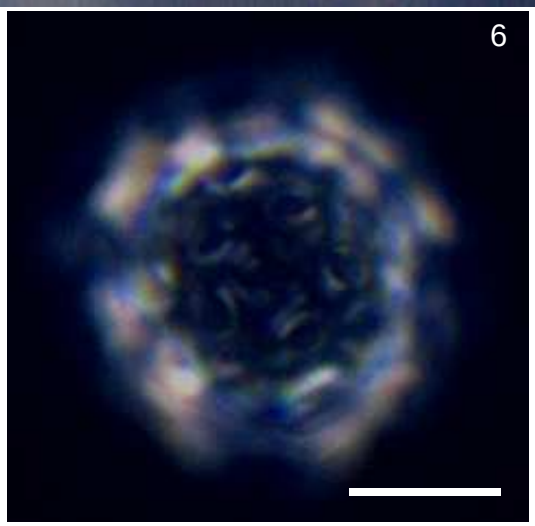

1


2


3

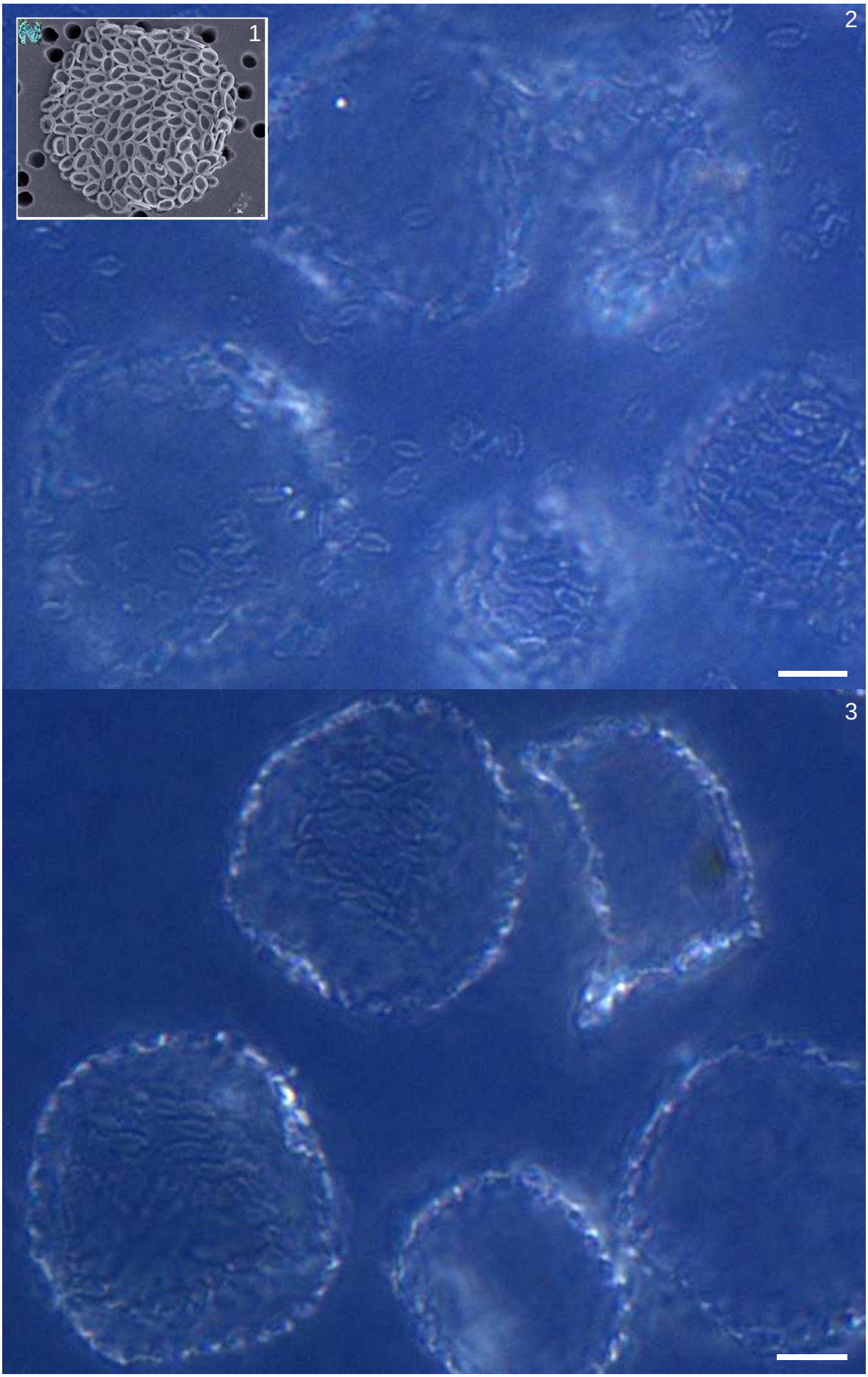

4


5


6

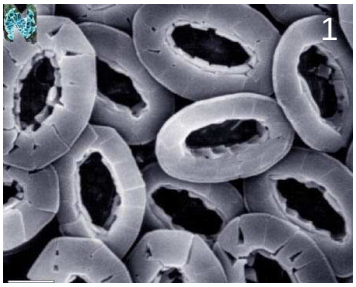
Pleurochrysidaceae

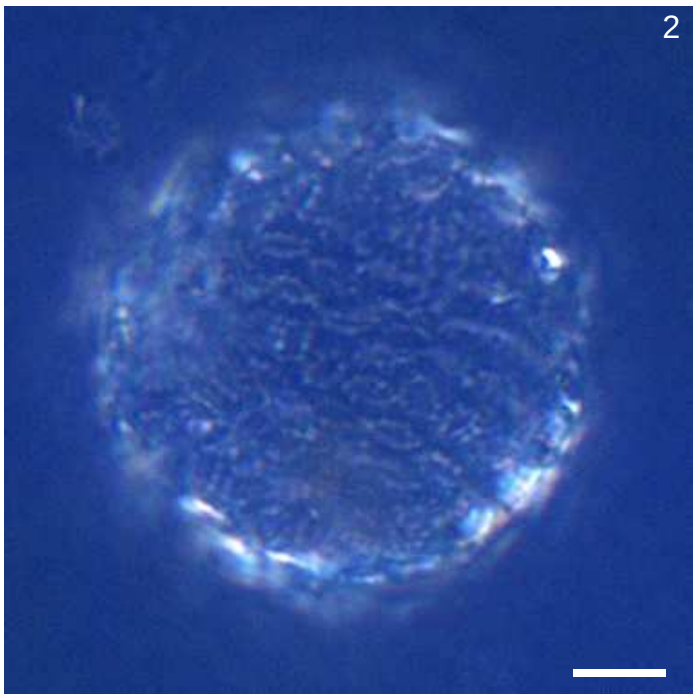
Plate 13. *Pleurochrysis carterae* (Braarud and Fagerland 1946) Christensen 1978 var. Carterae [Syracosphaera]
Scale bars = 5µm
1. *P. carterae*; collapsed coccosphere. (Plankton*Net; Authors: Jacqueline Fresnel; Copyright: Jacqueline Fresnel, U.Caen); SEM
2. *P. carterae*, cultured strain RCC1402

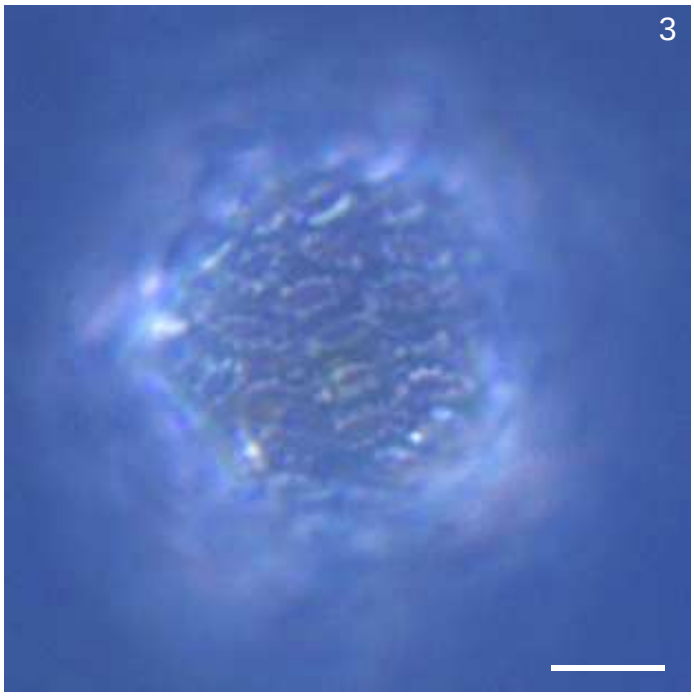

1
2
3

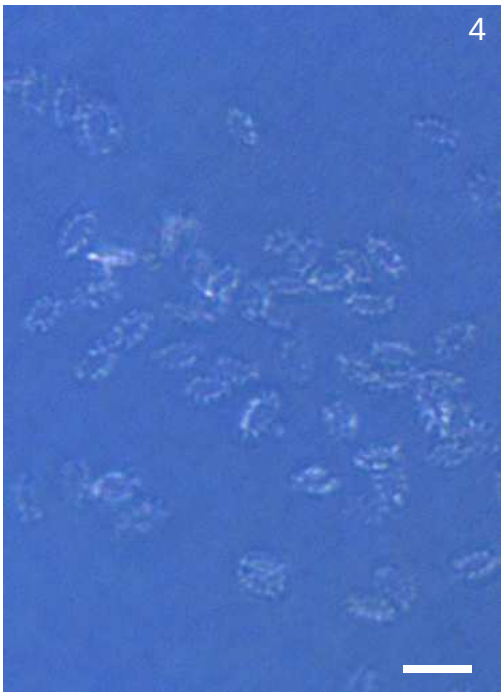
Pleurochrysidaceae

Plate 14. *Pleurochrysis placolithoides* Fresnel and Billard 1991
Scale bars = 5µm
1. *P. placolithoides*; collapsed coccosphere. (Plankton*Net; Authors: Jacqueline Fresnel; Copyright: Jacqueline Fresnel and Jeremy Young, U.Caen); SEM
2-4. *P. placolithoides,* cultured strain AC59



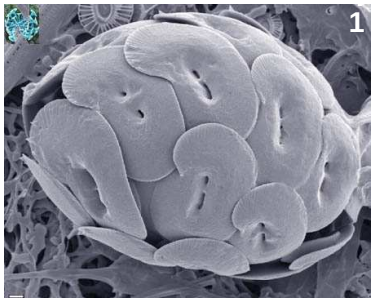


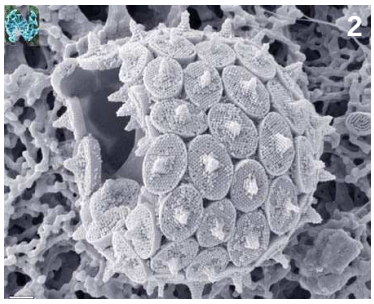


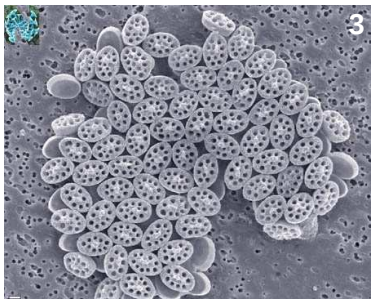


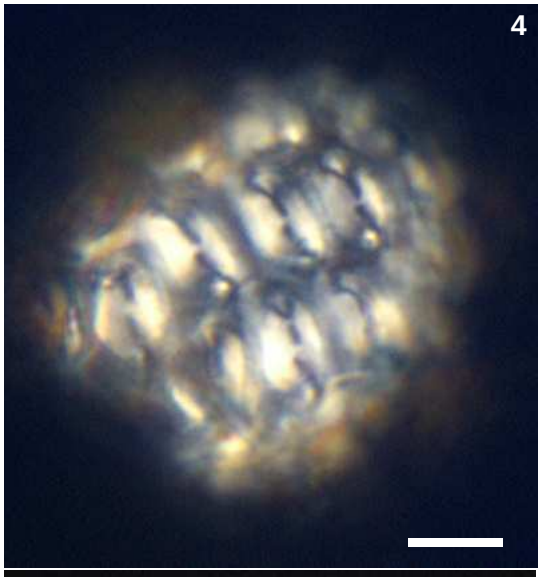
Helicosphaeraceae

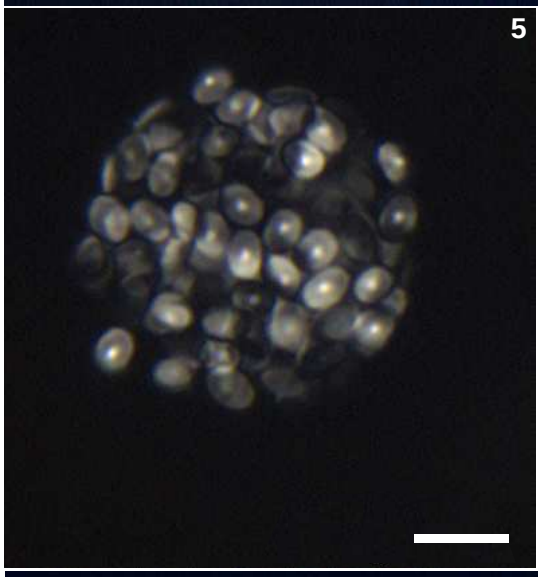
Plate 15. ***Helicosphaera*** Kampter 1954 (=*Helicopontosphaera* Hay & Mohler 1967)
Scale bars = 5µm
1. *H carteri* [HET] (Wallich 1877) Kampter 1954 [*Coccosphaera*] (Plankton*Net; Authors: Markus Geisen; Copyright: Jeremy Young, NHM); SEM.
2. *H carteri* [HOL solid] (‘*Syracolithus catilliferus*’) (Plankton*Net – image 16106; Authors: Claudia Sprengel and Jeremy Young; Copyright: Jeremy Young , NHM); SEM.
3. *H carteri* [HOL perforate] (‘*Syracolithus confusus*’) (Plankton*Net – image 16106; Authors: Richard Jordan and Jeremy Young; Copyright: Jeremy Young , NHM); SEM.
4. *H carteri* [HET]; 10º00 N - 29º47 W (surface) North Atlantic [AMT16 cruise, June 2005]; XPL
5. *H carteri* [HOL solid]; 38º18 N - 30º03 W (surface) North Atlantic [AMT16 cruise, June 2005]; XPL
6. *H carteri* [HOL perforate]; 38º18 N - 30º03 W (surface) North Atlantic [AMT16 cruise, June 2005]; XPL
7. *H carteri* combination coccosphere, bearing both heterococcoliths and holococcoliths [HET & HOL solid]; 38º18 N - 30º03 W (surface) North Atlantic [AMT16 cruise, June 2005]; XPL

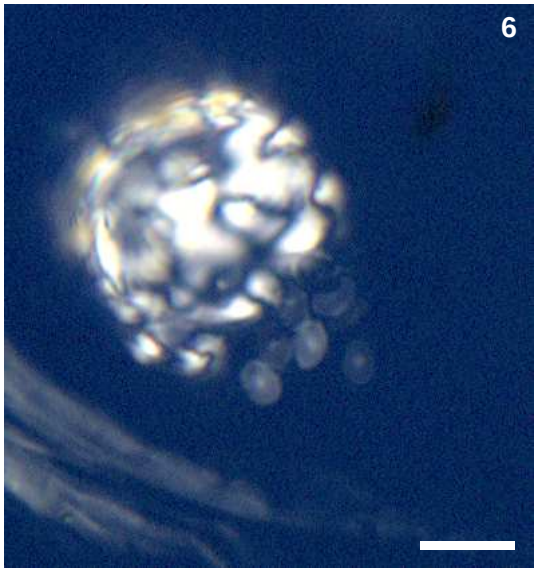


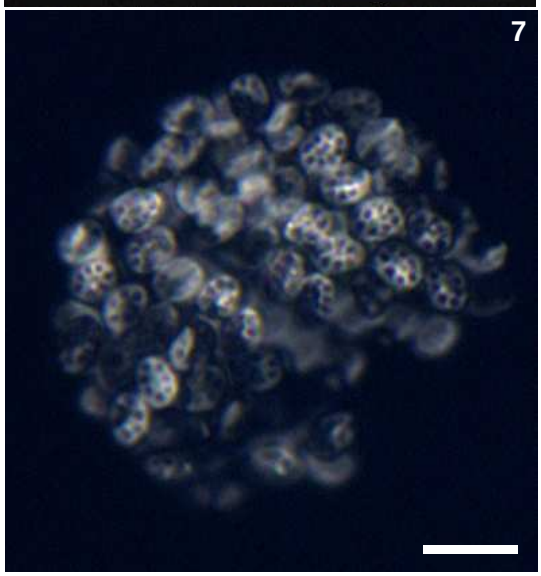






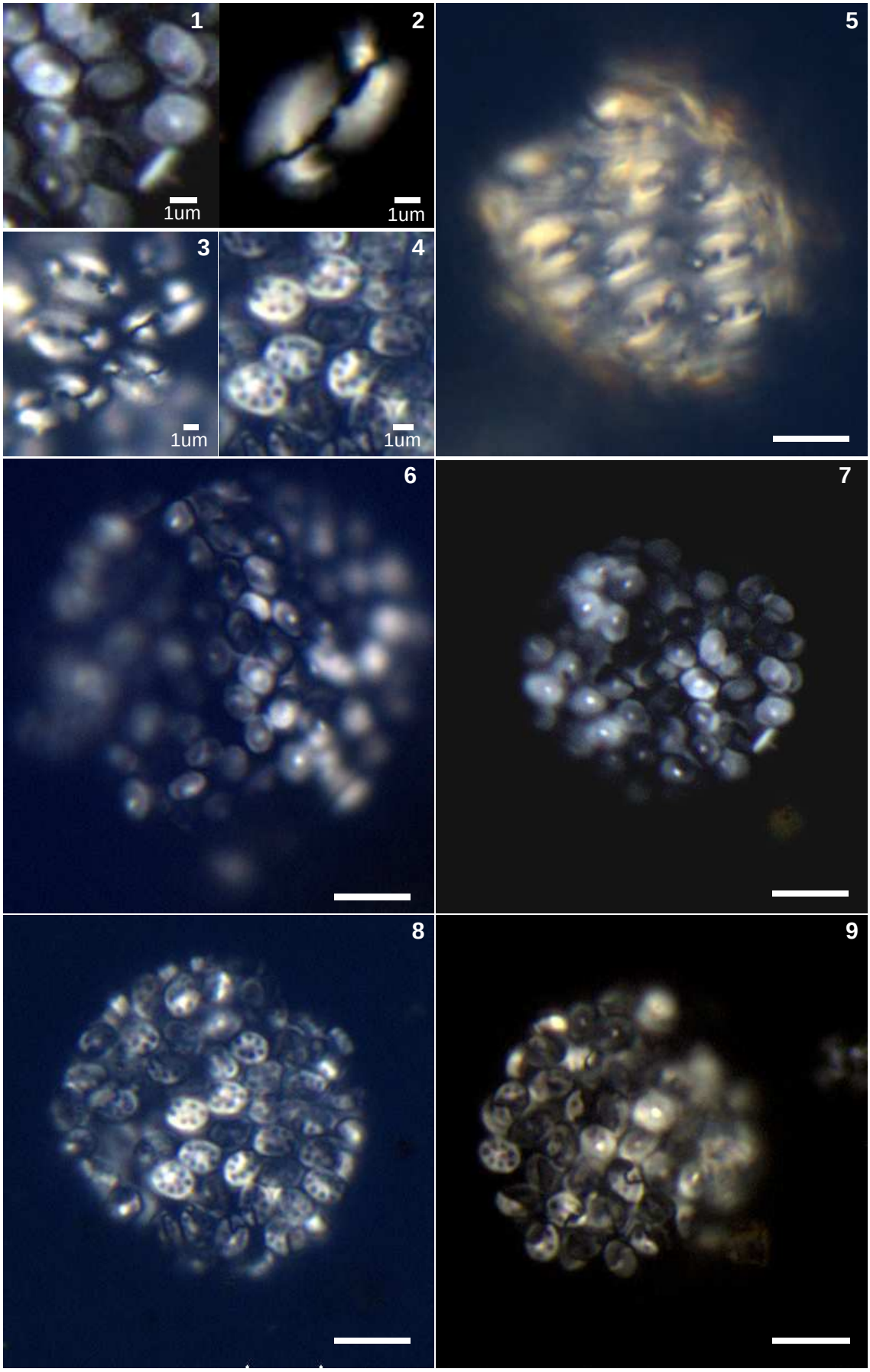






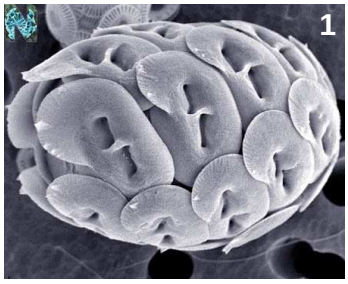
Helicosphaeraceae

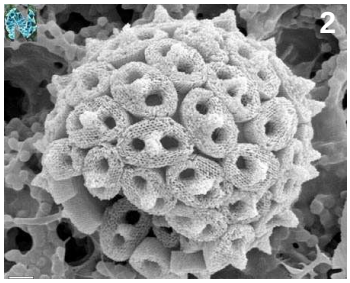
Plate 16. *Helicosphaera* Kampter 1954 (=*Helicopontosphaera* Hay & Mohler 1967)
Scale bars = 5µm (exception : = 1µm)
1. *H carteri* [HOL solid]; detail of holococcolith
2. *H carteri* [HET]; detail of heterococcolith
3. *H carteri* [HET]; detail of heterococcolith
4. *H carteri* [HOL perforate]; detail of holococcolith
5. *H carteri* [HET]; 10º00 N - 29º47 W (surface) North Atlantic [AMT16 cruise, June 2005]; XPL
6. *H carteri* [HOL solid]; 20º93 S - 24º59 W (surface) South Atlantic [AMT16 cruise, May 2005]; XPL
7. *H carteri* [HOL solid]; 38º18 N - 30º03 W (surface) North Atlantic [AMT16 cruise, June 2005]; XPL
8. *H carteri* [HOL perforate]; 31º22 N - 42º08 W (surface) North Atlantic [AMT16 cruise, June 2005]; XPL
9. *H carteri* combination coccosphere, bearing two types of holococcoliths [HOL solid & HOL perforate]; 38º18 N - 30º03 W (surface) North Atlantic [AMT16 cruise, June 2005]; XPL

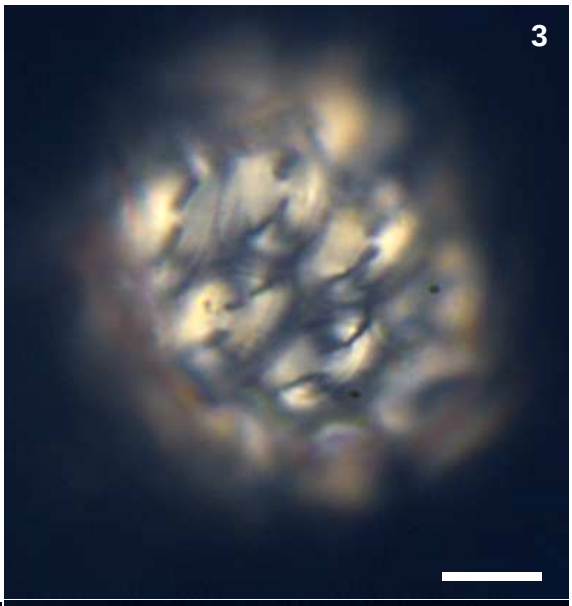

1
1um
2
1um
5
3
1um
4
1um
6
7
8
9

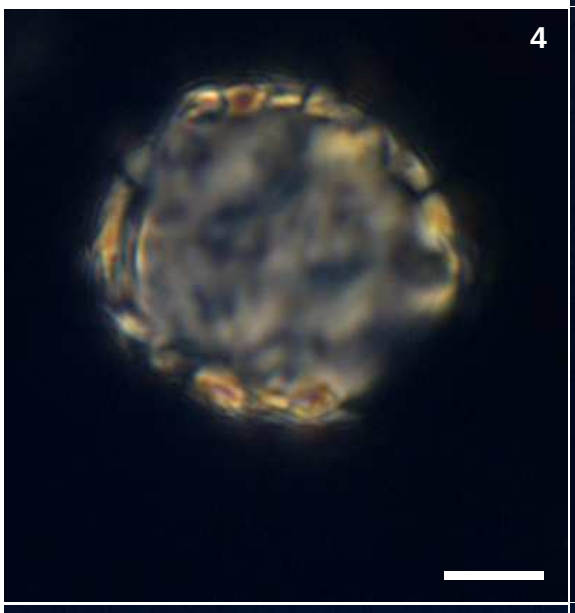
Helicosphaeraceae

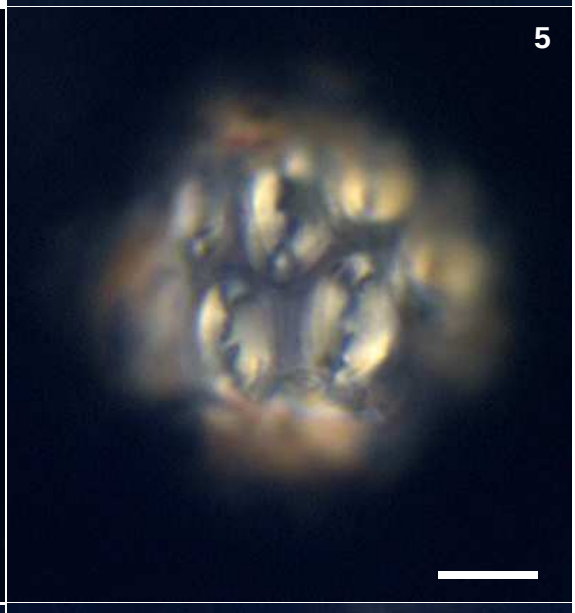
Plate 17. *Helicosphaera* Kampter 1954 (=*Helicopontosphaera* Hay & Mohler 1967)
Scale bars = 5µm
1. *H wallichii* (Lohmann 1902) Okada & McIntyre 1977 [*Coccolithophora*] (Plankton*Net; Authors: Markus Geisen; Copyright: Univ. Tsukuba); SEM.
2. *H wallichii* HOL solid ('*Syracolithus ponticuliferus*'), *Martine Couapel unpublished data*, (Plankton*Net – image 56438; Authors: Vita Pariente and Claire Findlay; Copyright: Jeremy Young , NHM); SEM.
3-5. *H wallichii* [HET]; 04º16 N - 27º01 W (surface) North Atlantic [AMT16 cruise, May 2005]; XPL
6-7. *H wallichii* [HOL]; 25º40 N - 37º40 W (surface) North Atlantic [AMT16 cruise, May 2005]; XPL

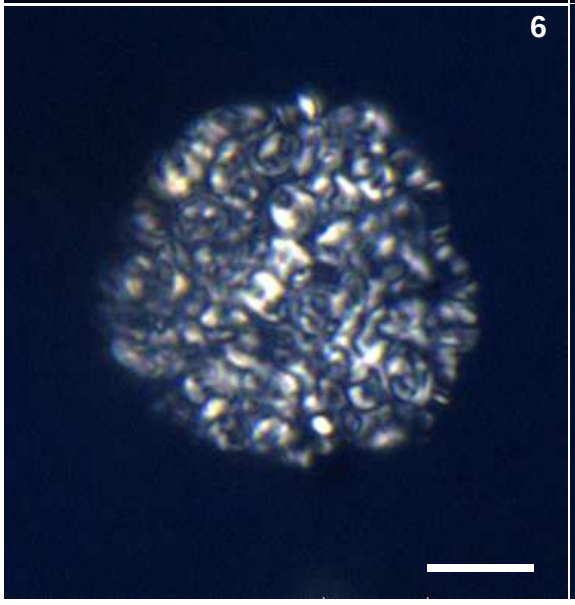

1

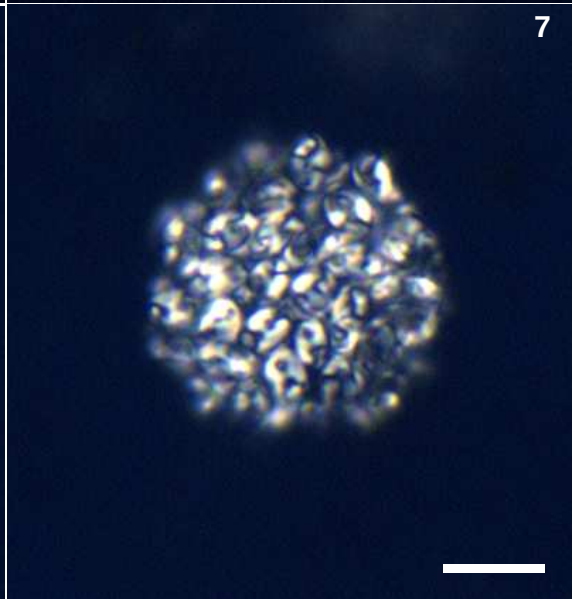

2


3


4

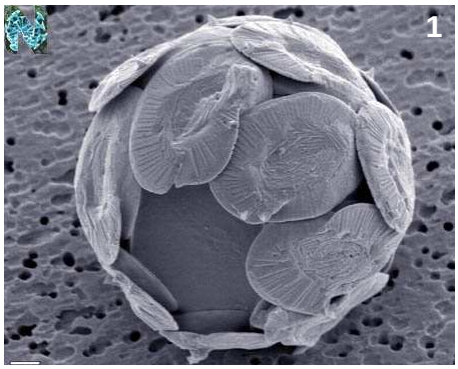

5

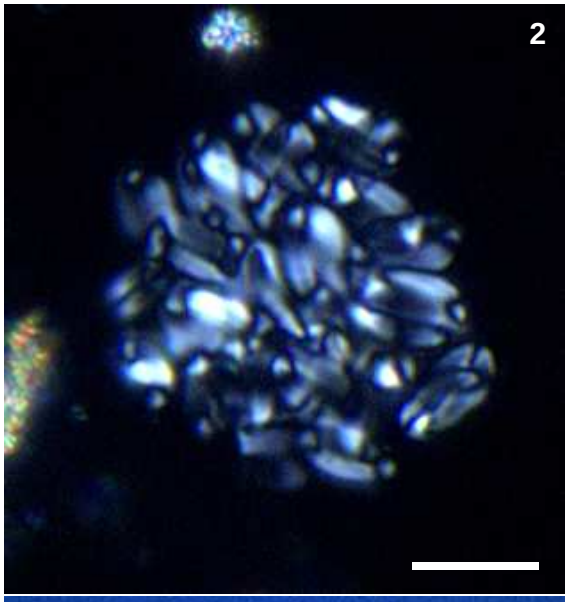

6


7

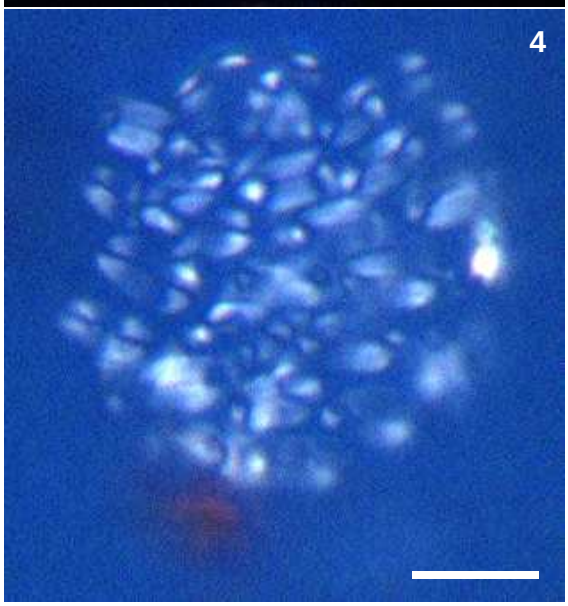
Helicosphaeraceae

Plate 18. *Helicosphaera hyalina* Gaarder 1970
Scale bars = 5µm
1. *H hyalina* (Plankton*Net; Authors: Ian Probert and Jeremy Young; Copyright: Jeremy Young ,NHM); SEM
2-4**.** *H hyalina* [HET]; 30º32’ N - 28’33’W Azores (surface) and 26º31 S - 17º13 W (surface) South Atlantic [AMT16 cruise, May 2005]; XPL and PhM


1

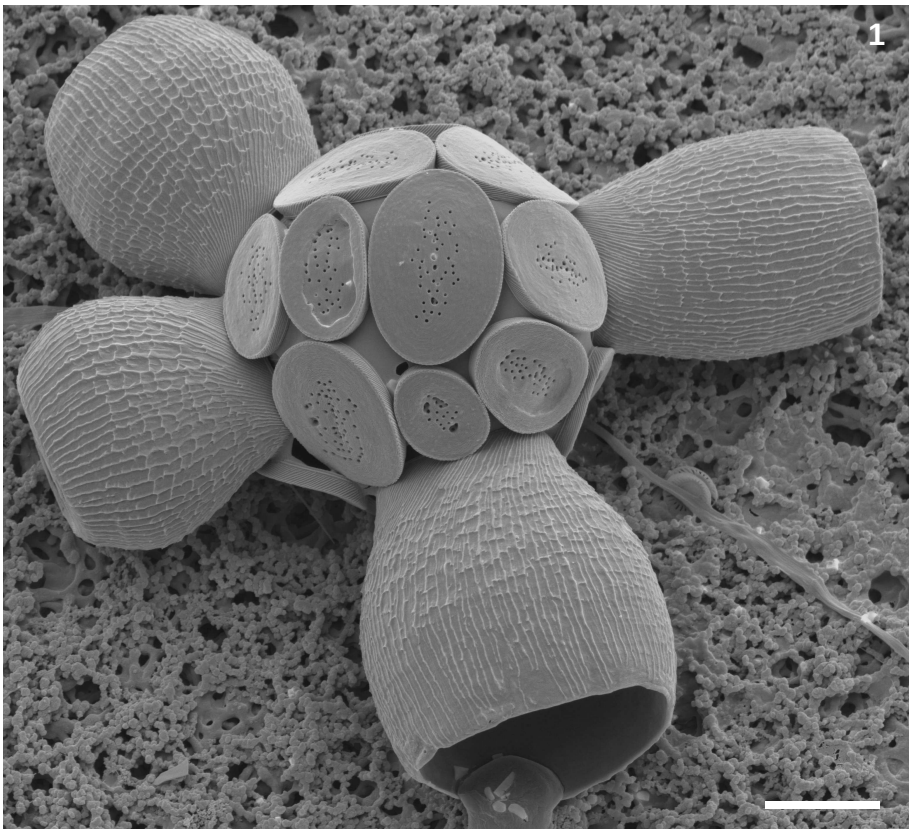

2

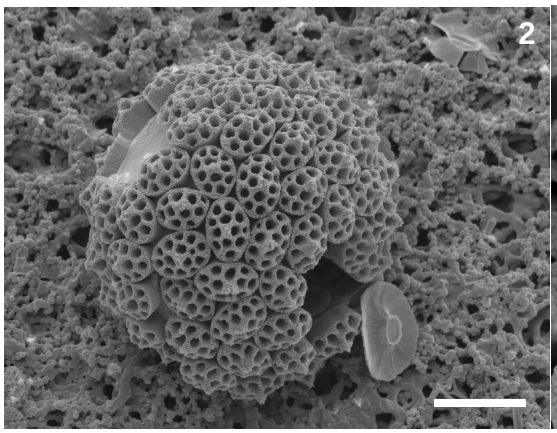

3

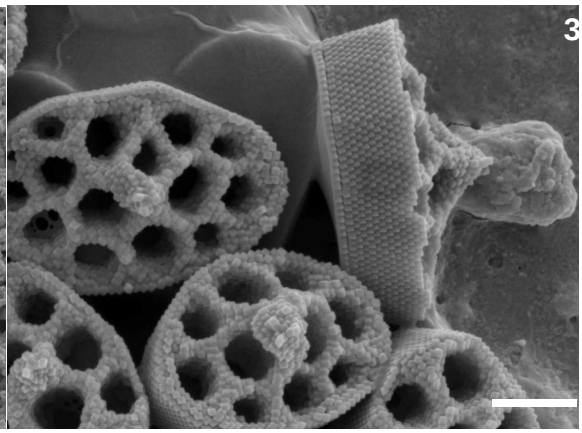

4

Pontosphaeraceae

Plate 19. ***Scyphosphaera*** Lohmann 1902
Scale bars = 5µm (exception : = 1µm)
1. *S apsteinii* [HET] (Authors: Jeremy Young, NHM); SEM
2. *S apsteinii* [HOL] (‘*Syracolithus schillerii’*) ; SEM
3. Detail of *S apsteinii* [HOL] holococcoliths; SEM

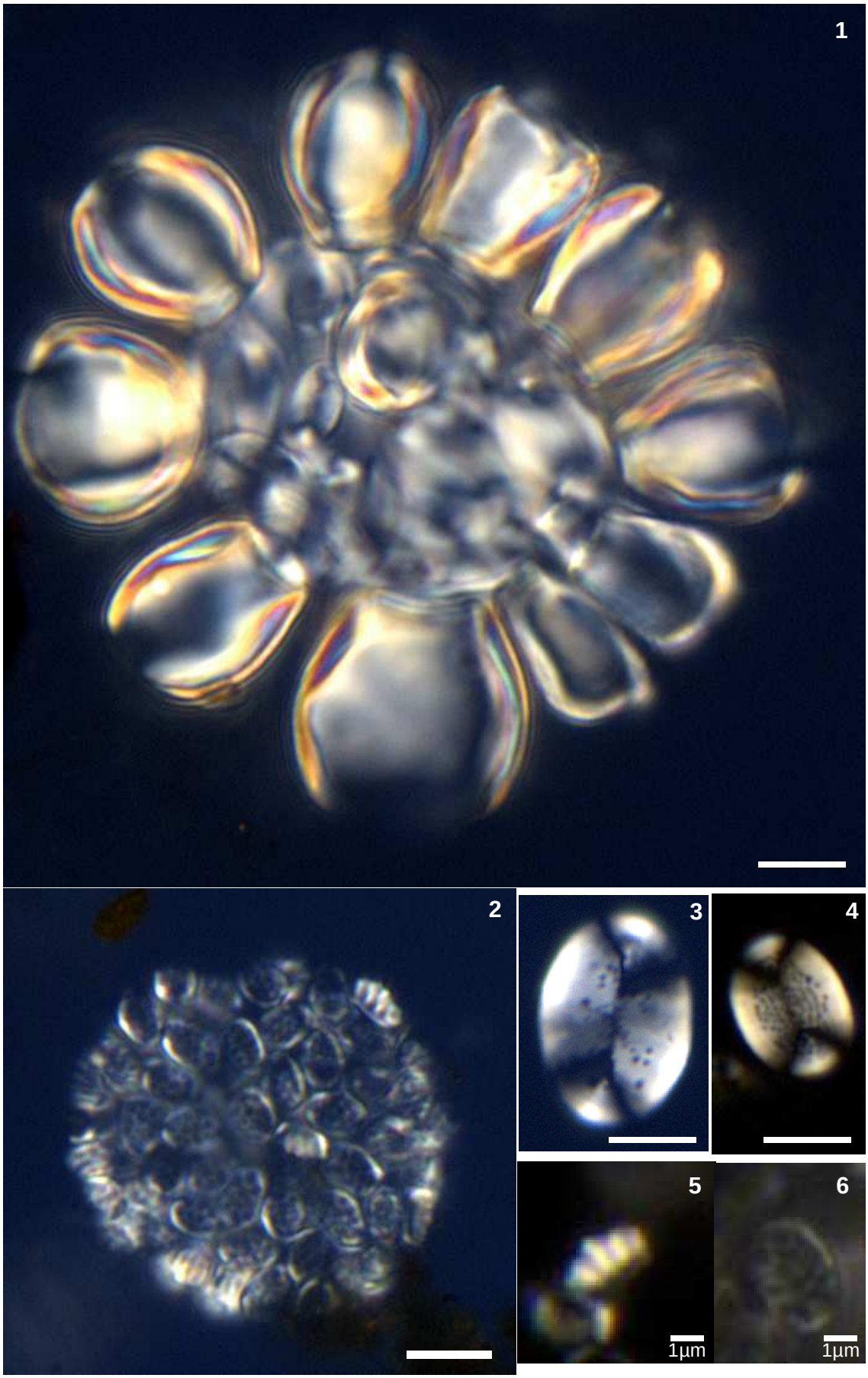






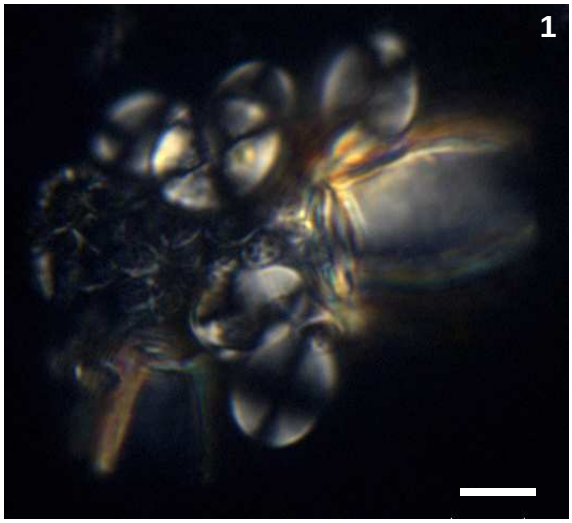
Pontosphaeraceae

Plate 20. ***Scyphosphaera*** Lohmann 1902
Scale bars = 5µm (exception : = 1µm)
1. *S apsteinii* [HET]; 26º31 S - 17º13 W (surface) South Atlantic [AMT16 cruise, May 2005]; XPL
2. *S apsteinii* [HOL] (‘*Syracolithus schillerii’*); 15º45 N - 32º35 W (surface) North Atlantic [AMT16 cruise, May 2005]; XPL
3-4. *S apsteinii* [HET] detached coccolith; XPL
5-6. *S apsteinii* [HOL] detached coccolith, side and top views; XPL

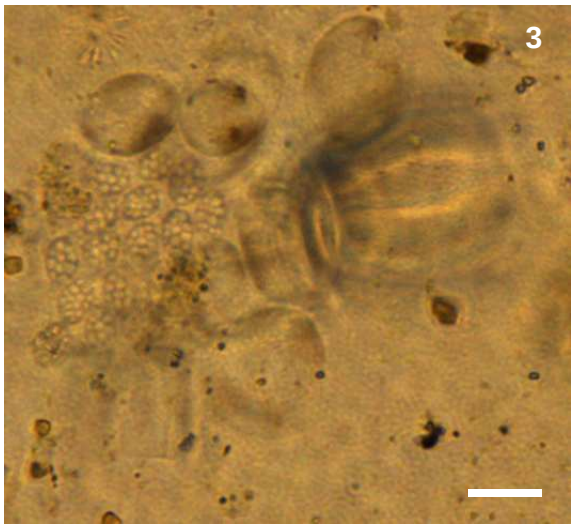

1
2
3
4
5
6
1µm
1µm

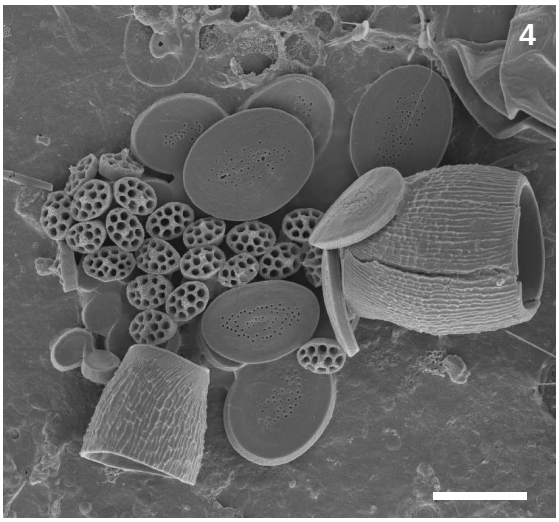
Pontosphaeraceae

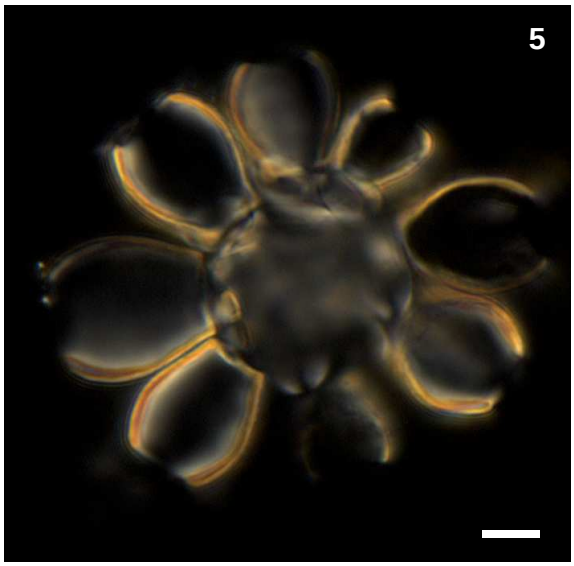
Plate 21. ***Scyphosphaera*** Lohmann 1902
Scale bars = 5µm
1-4. *S apsteinii* Lohmann 1902, combination coccosphere, bearing both heterococcoliths and holococcoliths [HET & HOL]; 38º18 N – 30º03 W (140m) North Atlantic [AMT16 cruise, May 2005]; XPL
5. *S apsteinii* [HET]; 36º27 N - 36º55 W (surface) North Atlantic [AMT16 cruise, June 2005]; XPL
6. *S apsteinii* [HET]; 36º31 S - 17º13 W (surface) South Atlantic [AMT16 cruise, May 2005]; XPL

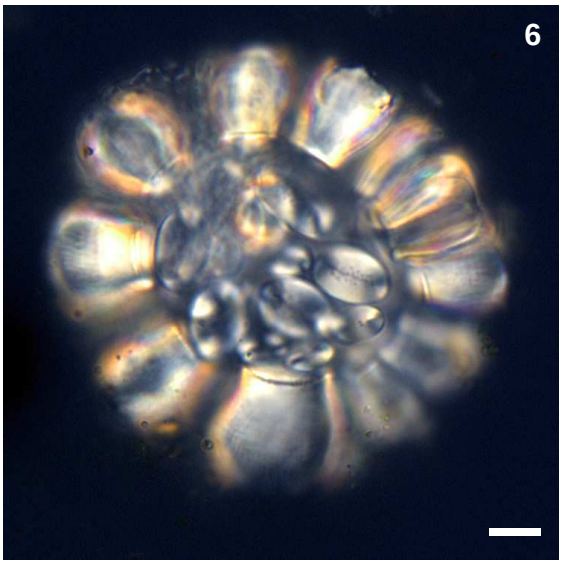

1


2


3

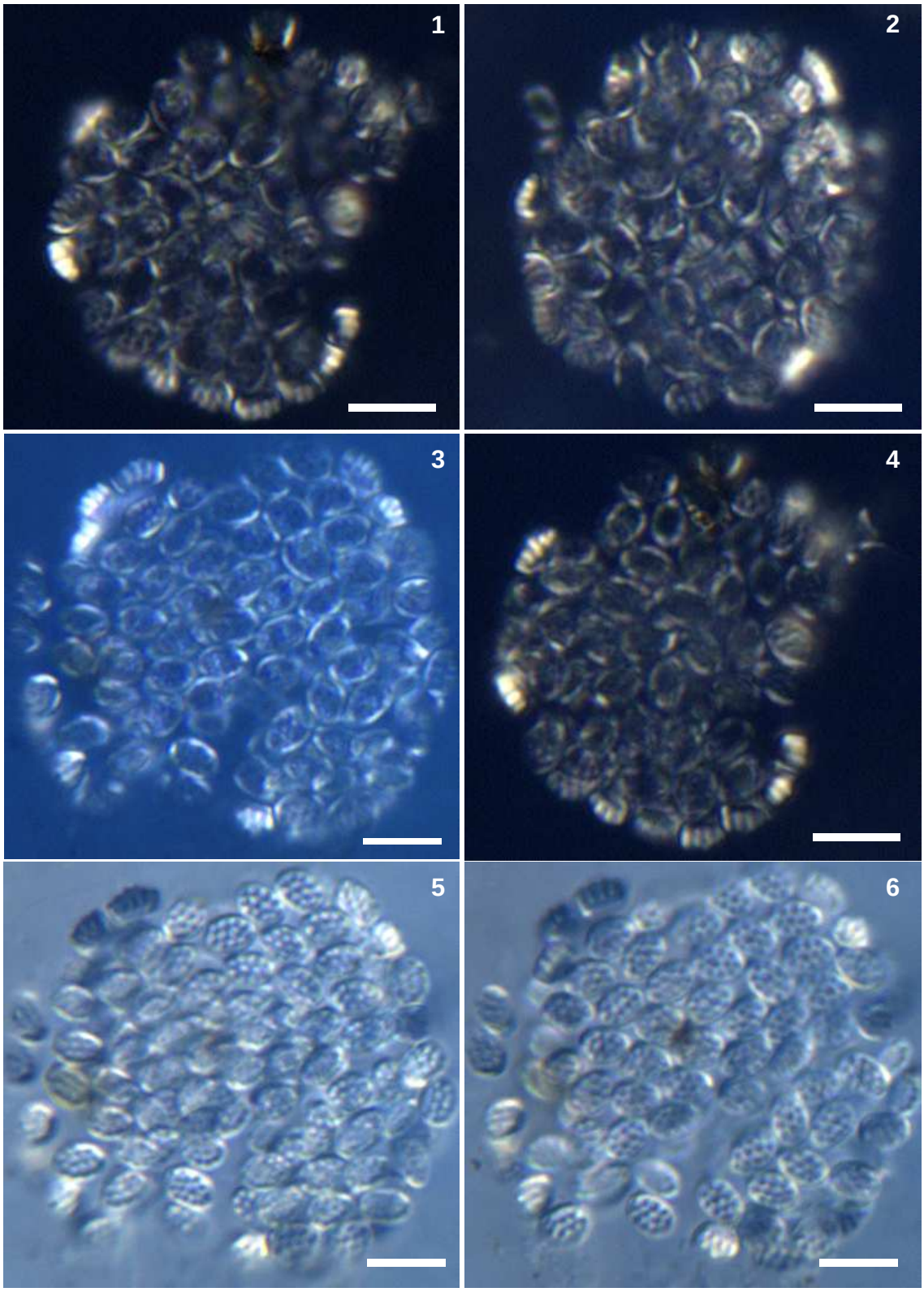

4


5


6

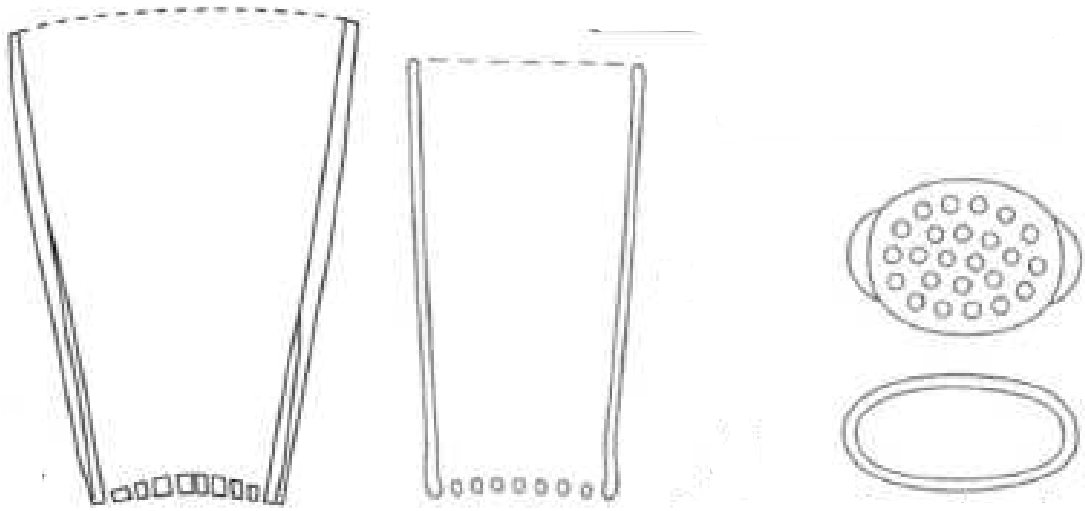
Pontosphaeraceae

Plate 22. ***Scyphosphaera*** Lohmann 1902
Scale bars = 5µm
1. *S apsteinii* [HOL]; 15º45 N – 32º35 W (surface) North Atlantic [AMT16 cruise, June 2005]; XPL
2-6. *S apsteinii* [HOL]; 29º09 N – 39º32 W (surface), 36º31 S - 17º13 W (surface) South/North Atlantic [AMT16 cruise, May 2005]; XPL


1
2
3
4
5
6

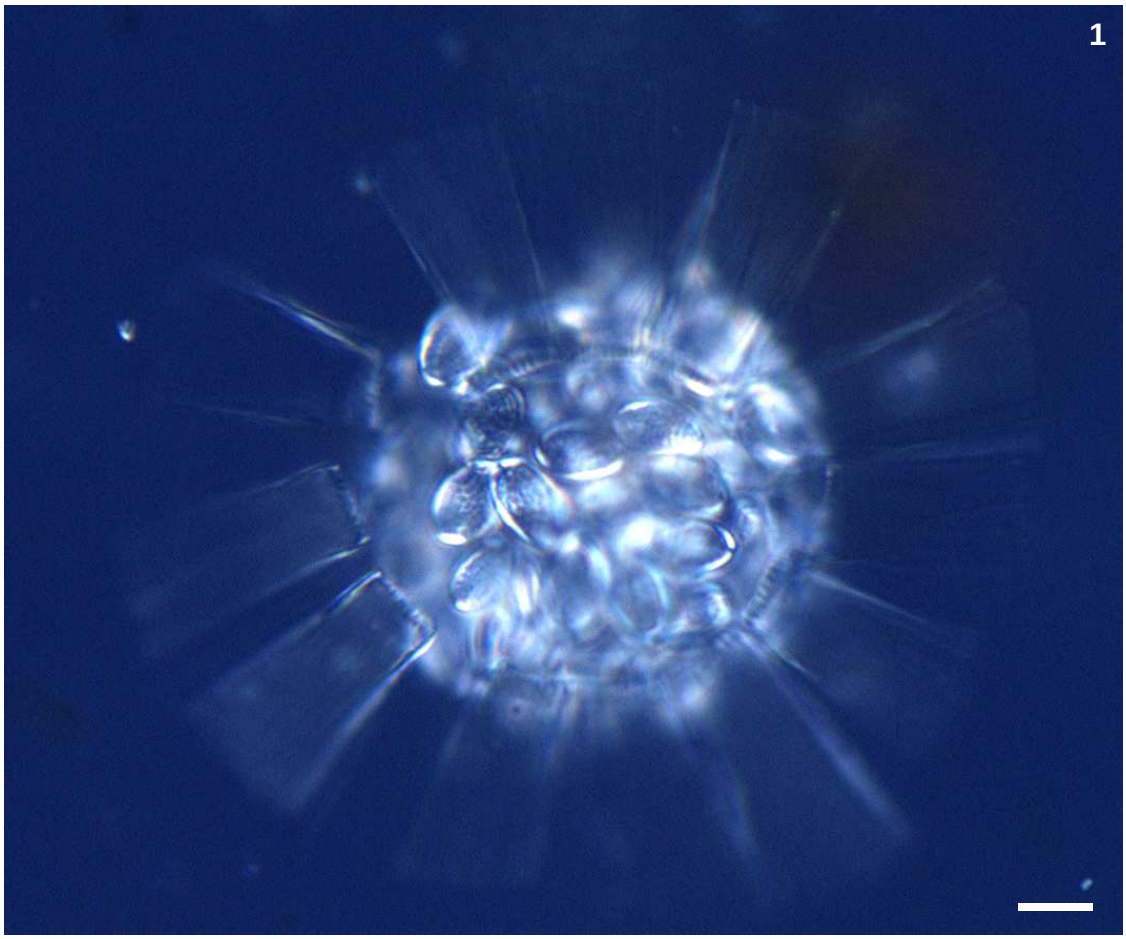
Pontosphaeraceae

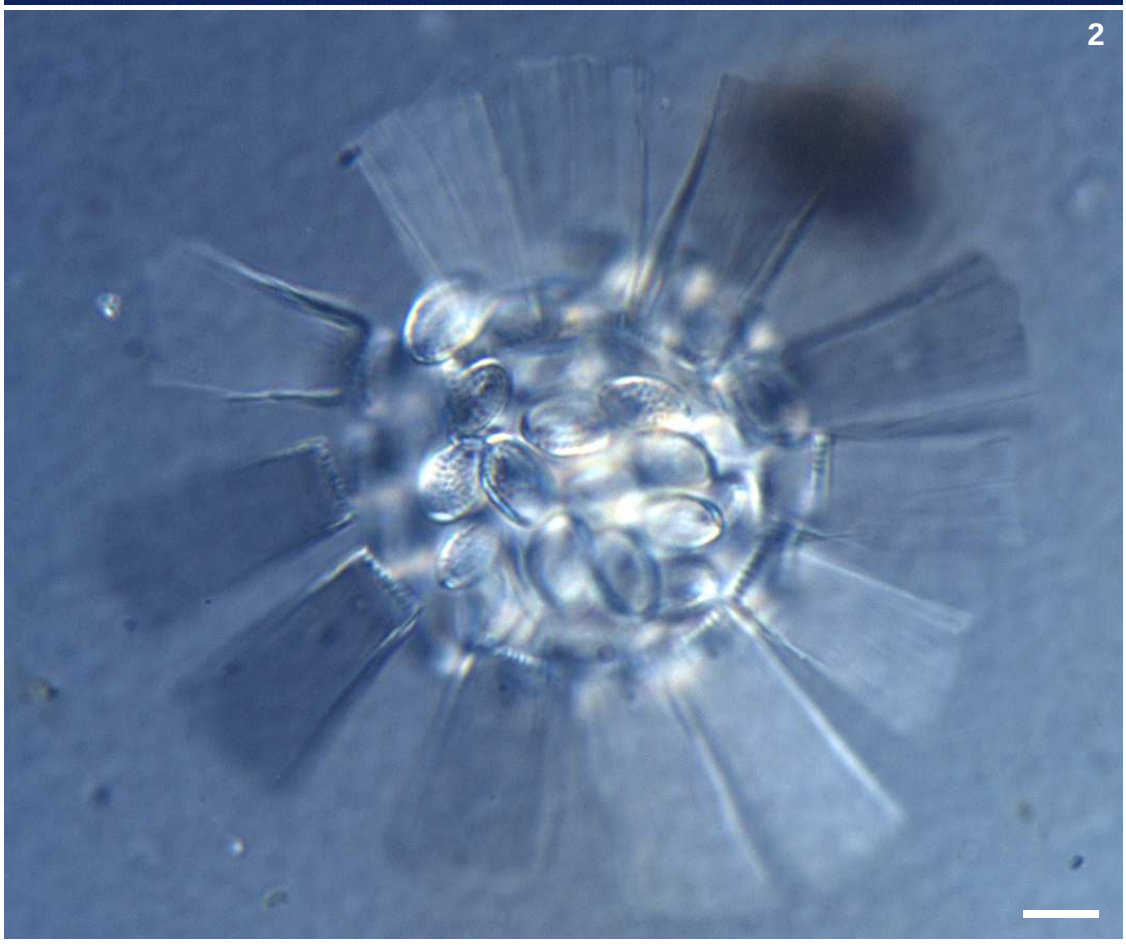
Plate 23. *Scyphosphaera* Lohmann 1902
Scale bars = 5µm
1. *S porosa* heterococcoliths; drawings published in Young 2008



Pontosphaeraceae

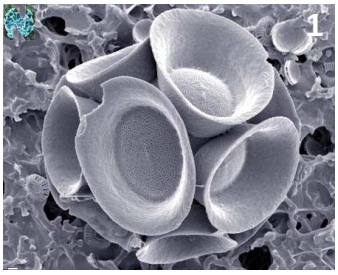
Plate 24. *Scyphosphaera* Lohmann 1902
Scale bars = 5µm
1-2. *S porosa* [HET]; 31º49’ S – 10º30’ E (96m), South Atlantic [AMT16 cruise, May 2005]; XPL and PhM

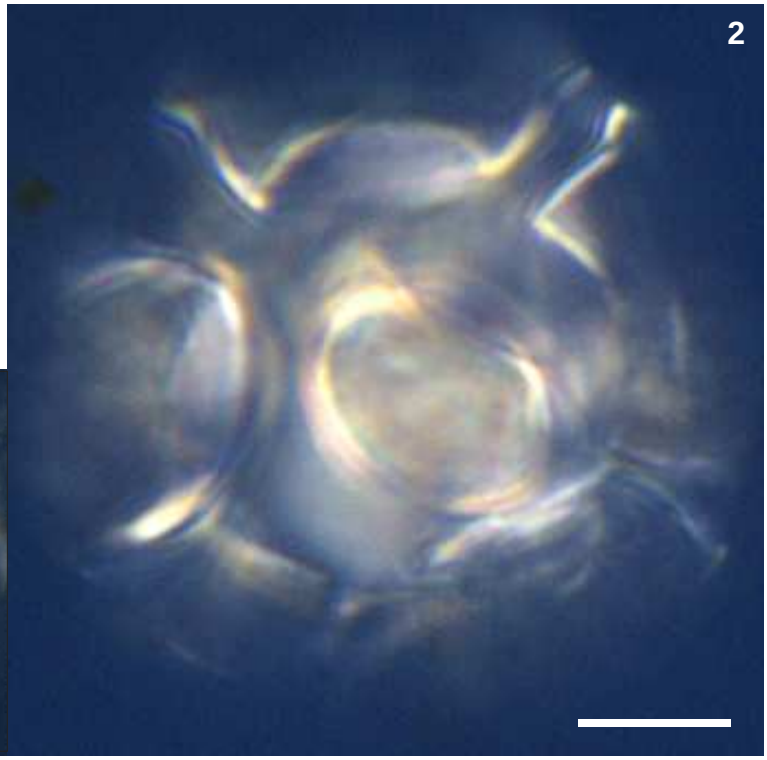

1

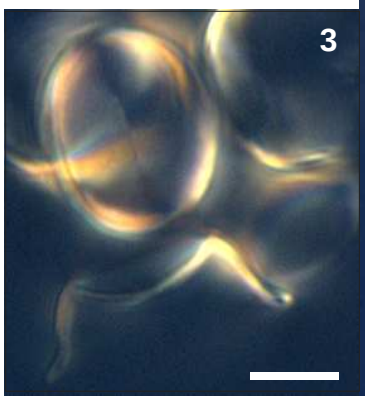

2

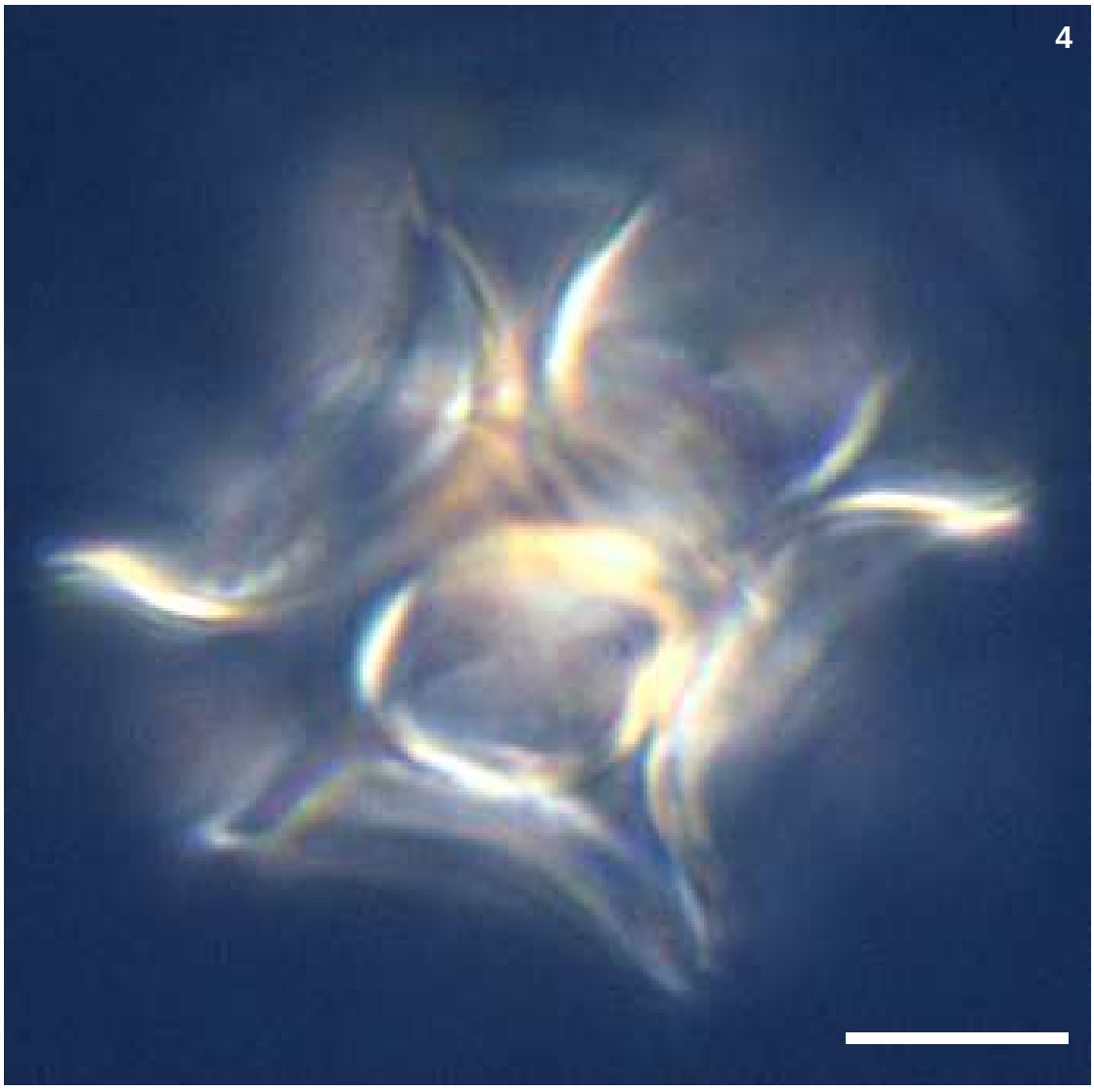
Pontosphaeraceae

Plate 25. *Pontosphaera* Lohmann 1902
Scale bars = 5µm
1. *P syracusana* Lohmann 1902 (Plankton*Net; Authors: Markus Geisen; Copyright: Jeremy Young, NHM); SEM
2 and 4. *P syracusana;* 36º31 S - 17º13 W (surface) South Atlantic [AMT16 cruise, May 2005]; XPL
3. *P syracusana* dispersed heterococcolith*;* 01º57 S - 24º59 W (surface) South Atlantic [AMT16 cruise, June 2005]; XPL


1

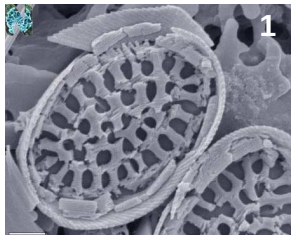

2

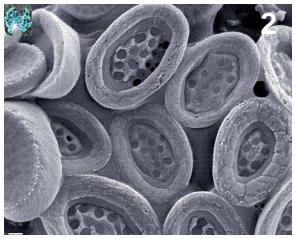

3

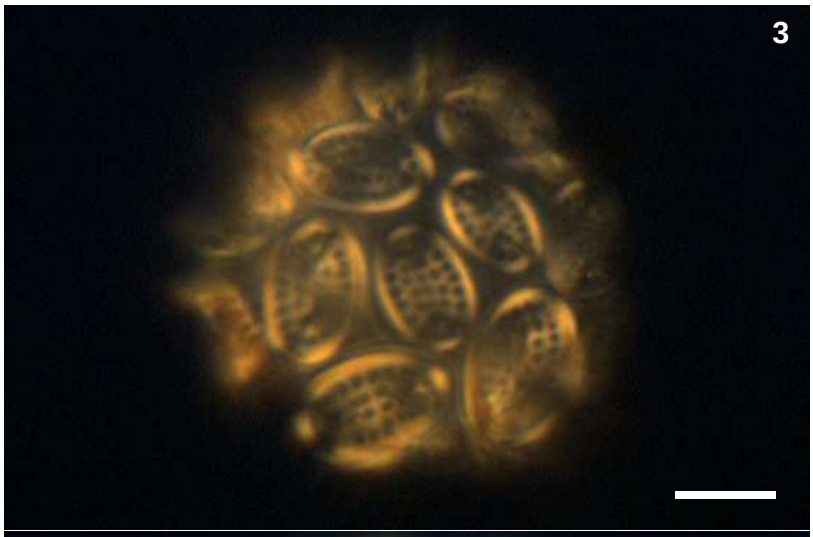

4

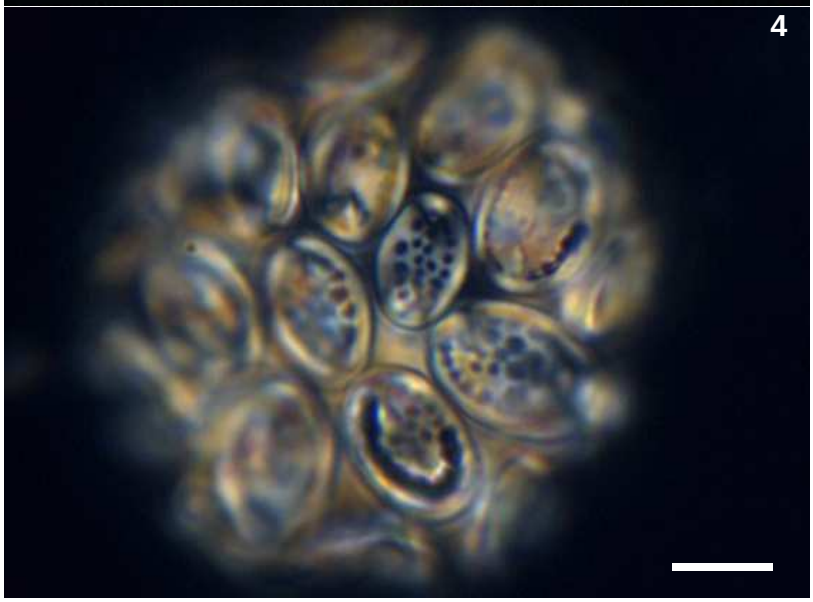
Pontosphaeraceae

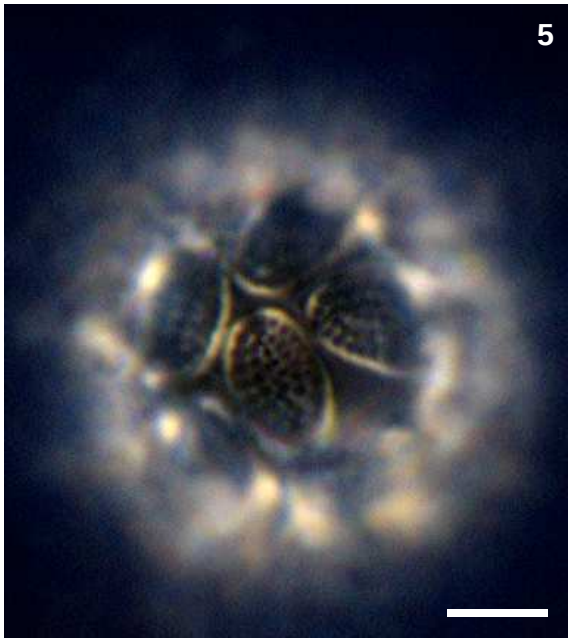
Plate 26. *Pontosphaera* Lohmann 1902
Scale bars = 5µm (exception : = 1µm)
1. *P multipora* [HET] (Kampter 1948) Roth 1970 [*Discolithina*], coccolith distal view (Plankton*Net; Authors: Vita Pariente and Jeremy Young; Copyright: Jeremy Young, NHM); SEM
2. *P multipora* [HET], coccolith distal view (Plankton*Net; Authors: Vita Pariente and Claire Findlay; Copyright: Jeremy Young, NHM); SEM
3. *P multipora* [HET]; 27º49 S - 10º30 W (surface) South Atlantic [AMT16 cruise, May 2005]; XPL
4. *P multipora* [HET]; 26º31 S - 17º13 W (surface) South Atlantic [AMT16 cruise, May 2005]; XPL
5-7. *P multipora* [HET]; 20º11' S – 24º59' W (surface) South Atlantic [AMT16 cruise, May 2005]; XPL

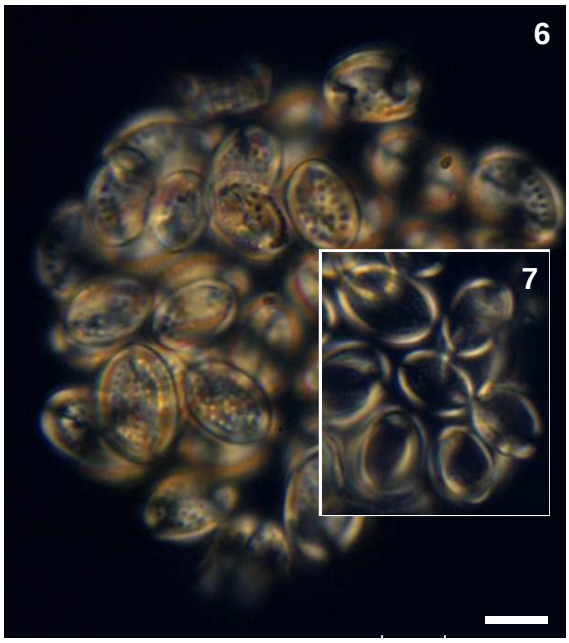

1


2


3

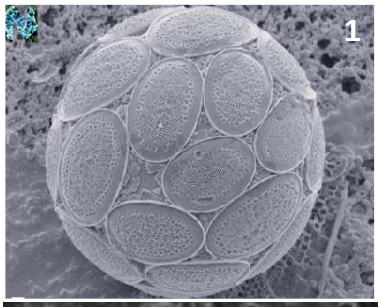

4

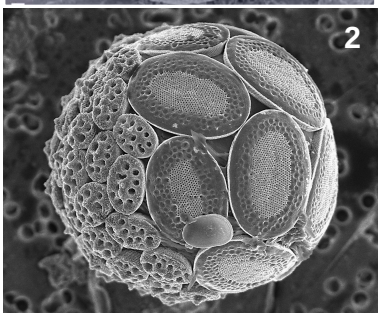

5

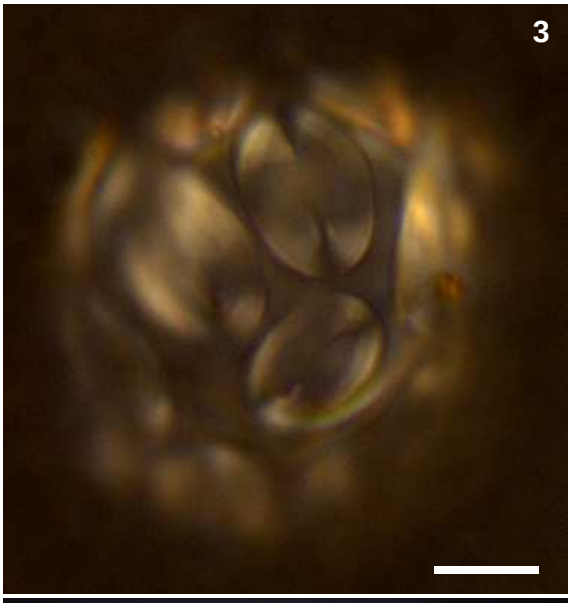

6
7

Pontosphaeraceae

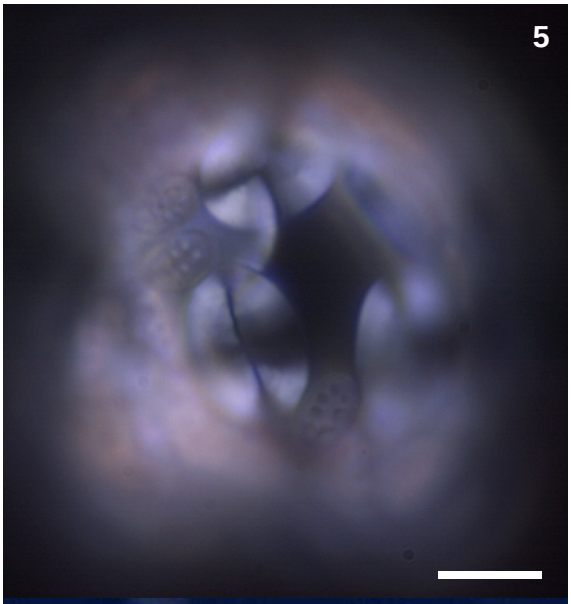
Plate 27. *Pontosphaera* Lohmann 1902
Scale bars = 5µm
1. *P japonica* [HET] (Takayama 1967) Nishida 1971 [*Discolithina*] (Plankton*Net; Authors: Jeremy Young; Copyright: Jeremy Young, NHM); SEM
2. *P japonica* – '*Syracolithus*' like holococcolithophore combination coccosphere [HET & HOL], gulf of Naples (Authors: Jeremy Young and Isabella Percopo, NHM); SEM
3. *P japonica* [HET]; 01º37 S - 24º59 W (surface) South Atlantic [AMT16 cruise, June 2005]; XPL
4. *P japonica* [HOL]; 39°30' N 13°30' E Tyrrhenian Sea [November 2006] (Author: Jeremy Young); PhM
5. *P japonica* combination coccosphere [HET & HOL]; gulf of Naples (Author: Jeremy Young); XPL
6. *P japonica* [HOL]; 39°30' N 13°30' E Tyrrhenian Sea [November 2006] (Author: Jeremy Young); XPL
7. *P japonica* [HOL], broken specimen; 18º57' N - 34º12' W (surface) North Atlantic [AMT16 cruise, May 2005]; XPL

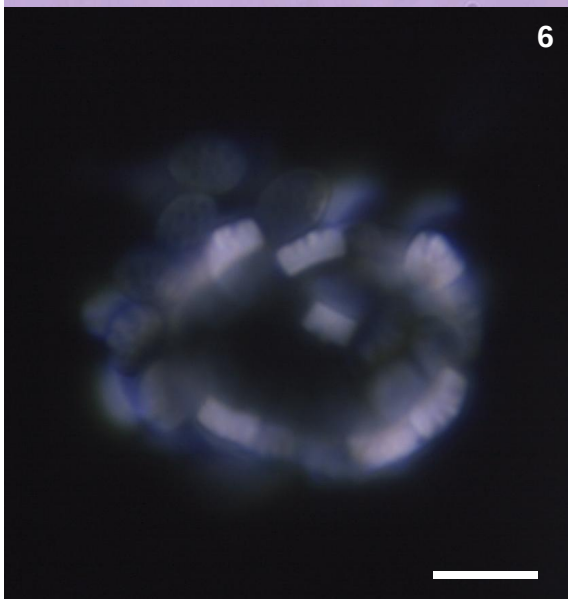

1

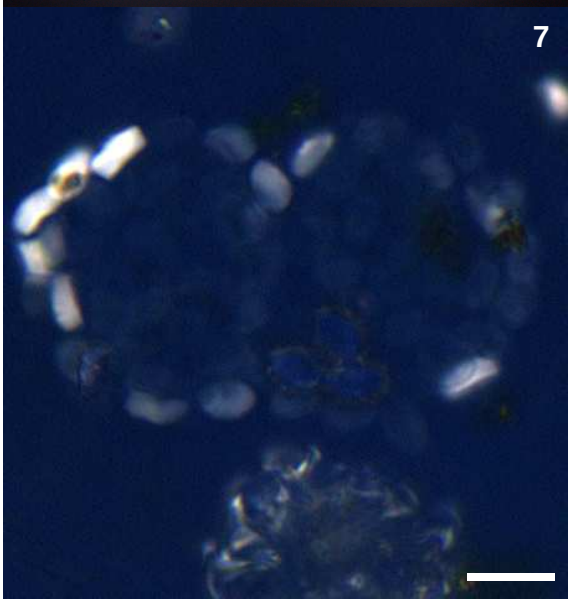

2


3


4

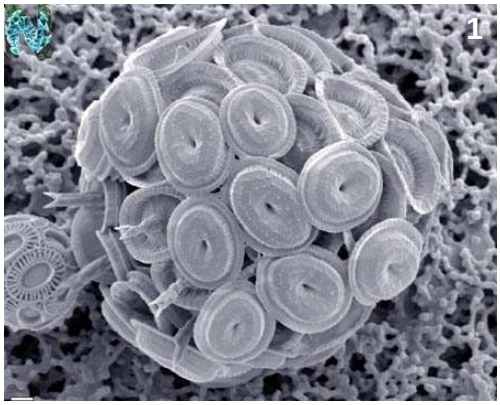

5

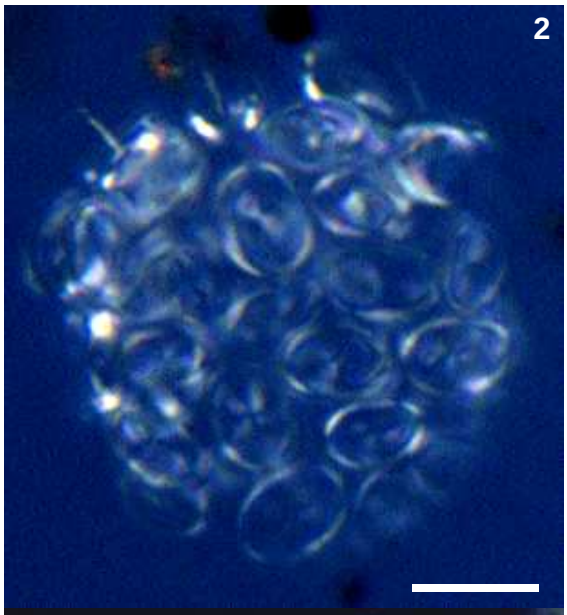

6

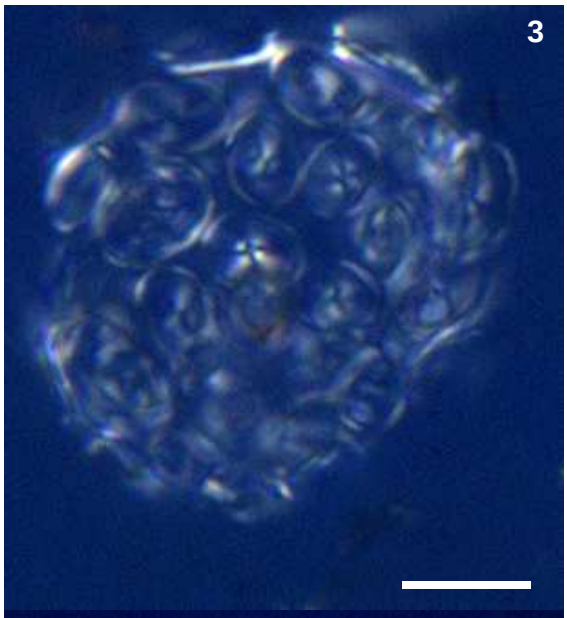

7

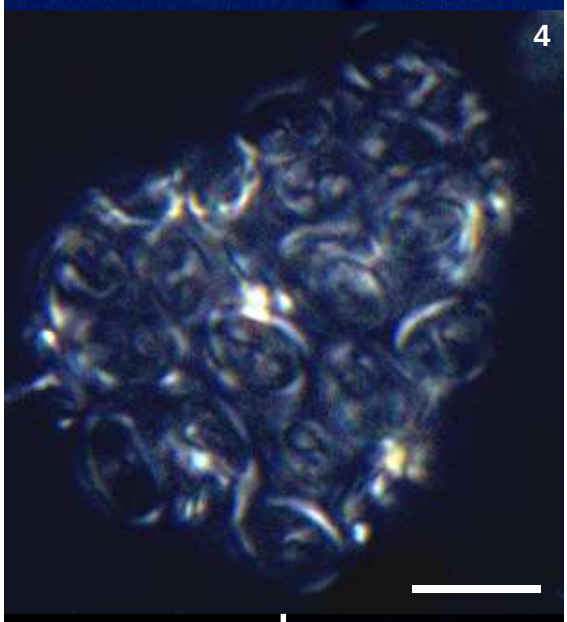
Syracosphaeraceae

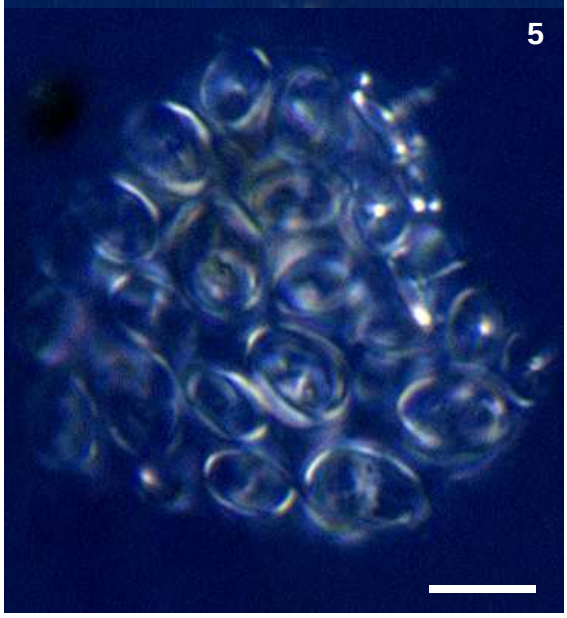
Plate 29. *Syracosphaera pulchra* Lohmann 1902
1. *S pulchra* [HET] (Plankton*Net; Authors: Claudia Sprengel and Jeremy Young; Copyright: Jeremy Young, NHM); SEM
2 and 5. *S pulchra* [HET]; 20º11’ S - 24º59’ W (surface) South Atlantic [AMT16 cruise, June 2005]; XPL
3. *S pulchra* [HET]; 04º16’ N - 27º01’ W (surface) South Atlantic [AMT16 cruise, June 2005]; XPL
4. *S pulchra* [HET]; 22º52 S - 24º59 W (surface) South Atlantic [AMT16 cruise, May 2005]; XPL
6-7. *S pulchra* [HET]; 30º32’ N - 28’33’W Azores , Faial (surface) [May 2008]; XPL

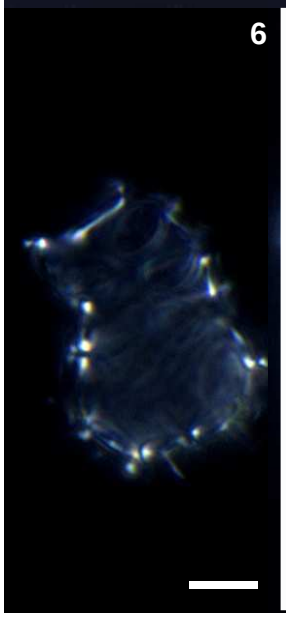

1


2


3

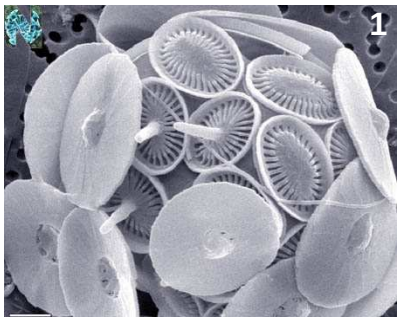

4

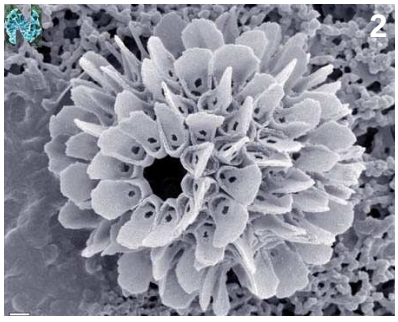

5

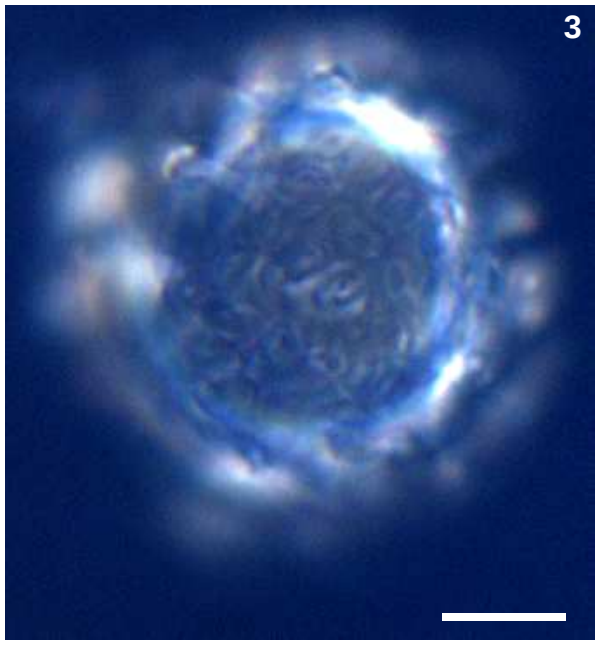

6

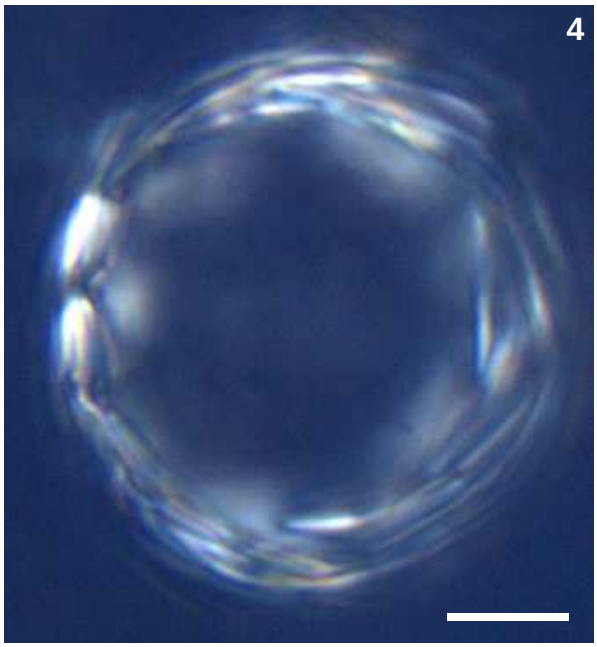

7

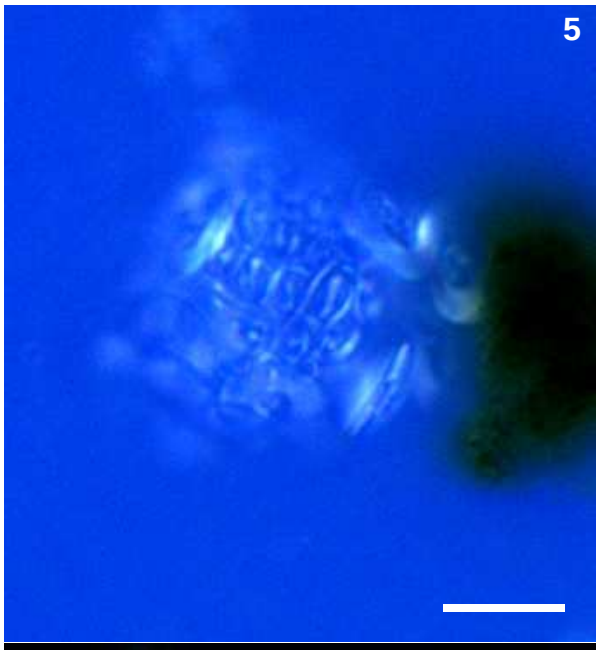
Plate 29. *Syracosphaera anthos* (Lohman 1912) Janin 1987 [Deutschlandia]
1. *S anthos* [HET] Lohmann 1902 (Plankton*Net; Authors: Markus Geisen and Jeremy Young; Copyright: Jeremy Young, NHM); SEM
2. *S. anthos* [HOL] (*Periphyllophora mirabilis*) (Cros e tal. 2000) (Plankton*Net; Authors: Markus Geisen and Jeremy Young; Copyright: Jeremy Young, NHM); SEM
3-5. *S anthos* [HET]; 31º 49'S - 01º30'E South Atlantic [AMT16 cruise, May 2005] (surface water); XPL
6-7. *S. anthos* [HOL] 30º32' N - 28'33'W Azores (surface) and 26º31 S - 17º13 W (surface) South Atlantic [AMT16 cruise, May 2005]; XPL

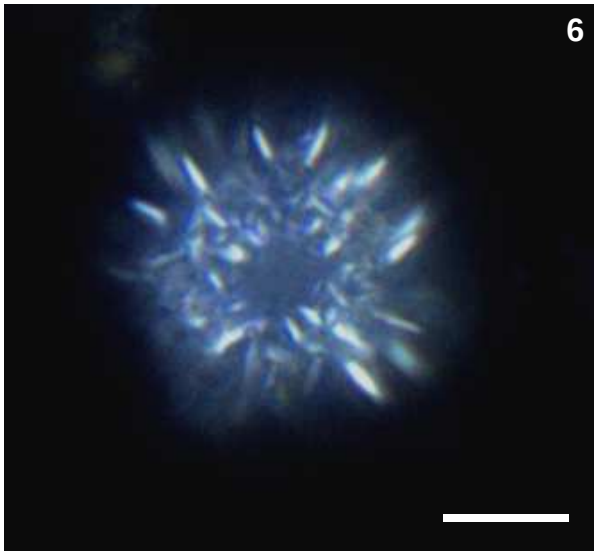

1

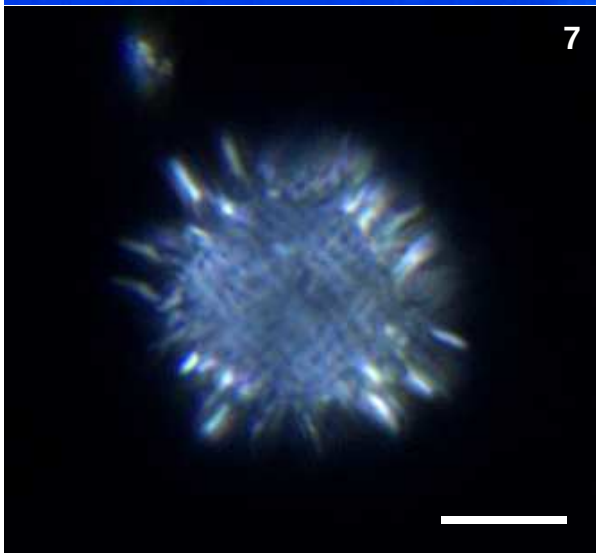

2


3


4

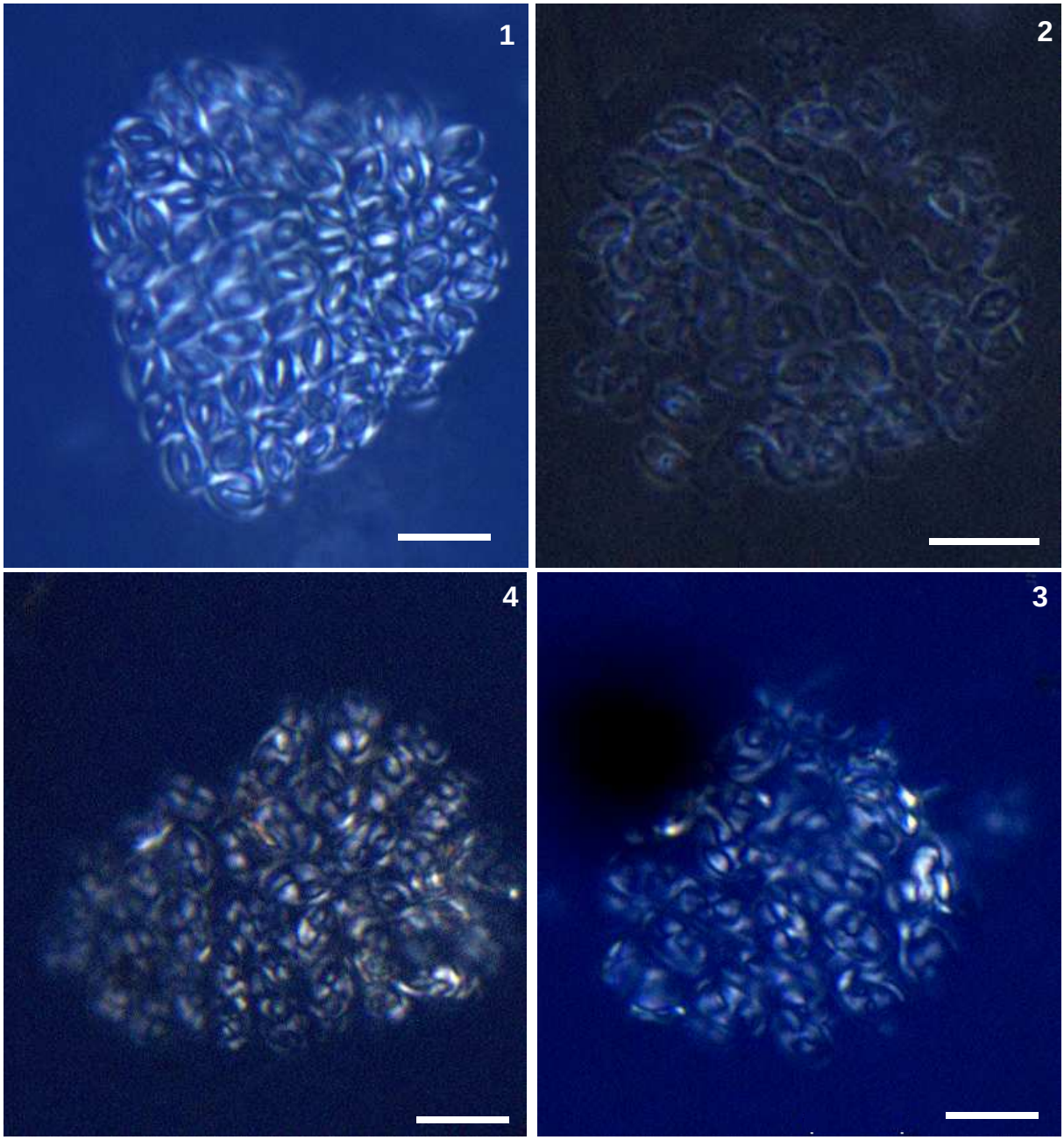

5


6


7

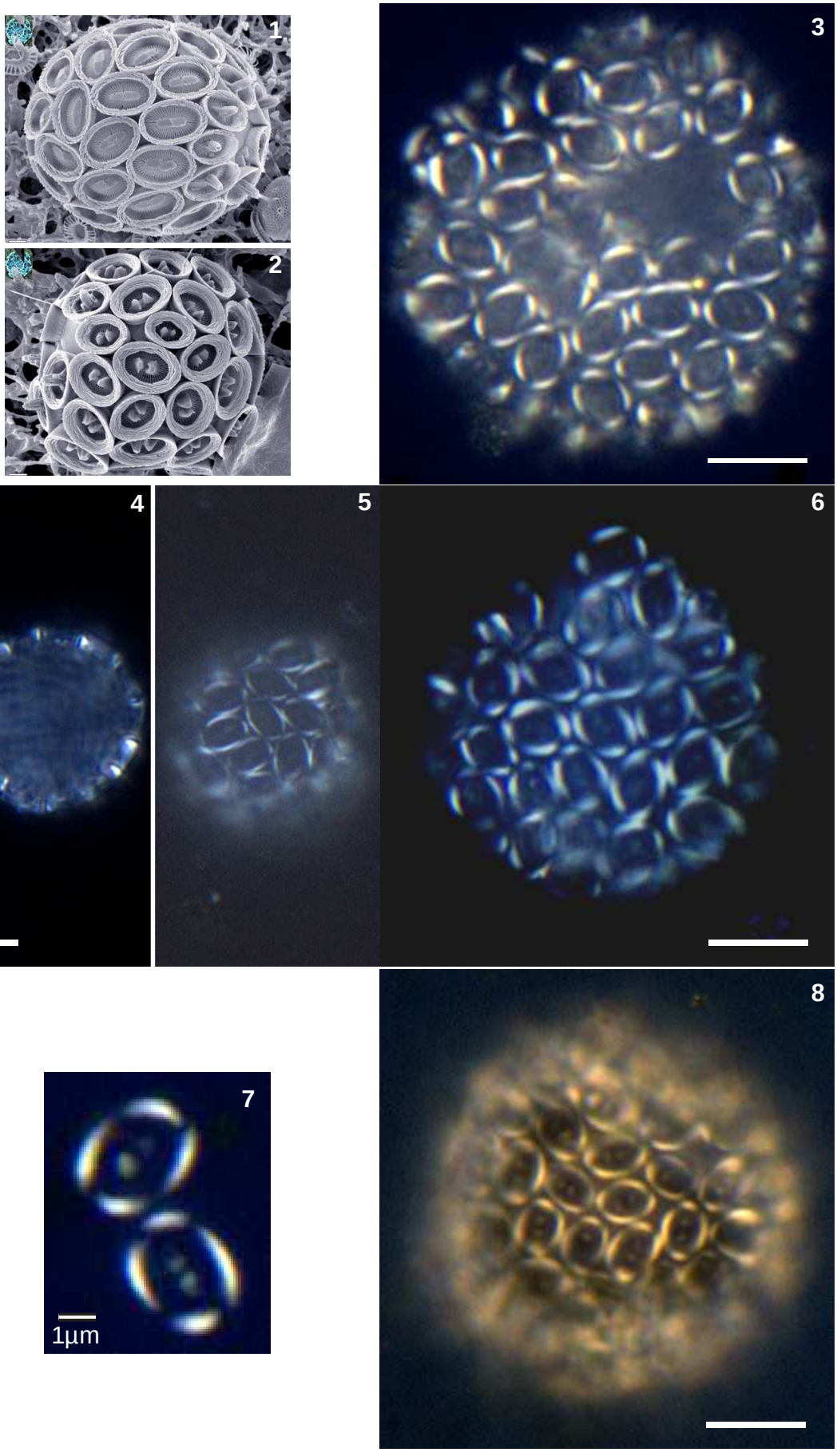
Syracosphaeraceae

Plate 30. *Syracosphaera* (Lohman 1912)
1. *Syracosphaera* [HET] – 1) Maybe *S. Lamina*
2. *Syracosphaera* [HET]
3-4. *Syracosphaera* ssp. [HET]; 31º 49’S - 01º30’E South Atlantic [AMT16 cruise, May 2005] (surface water); XPL


1
2
4
3

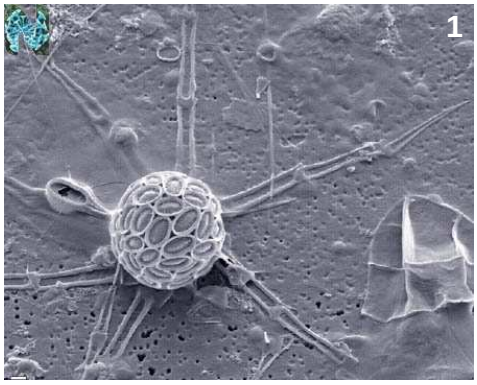
Syracosphaeraceae

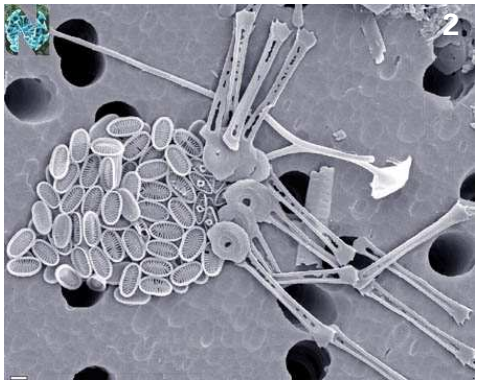
Plate 31. *Coronosphaera* Gaarder *in* Gaarder & Heimdal 1977
Scale bars = 5µm
1. *C mediterranea* [HET] (Lohmann 1902) Gaarder *in* Gaarder & Heimdal 1977 [*Syracosphaera*](Plankton*Net; Authors: Claudia Sprengel and Jeremy Young; Copyright: Jeremy Young, NHM); SEM
2. *C binodata* [HET] (Plankton*Net; Authors: Markus Geisen; Copyright: Jeremy Young, NHM); SEM
3. *C mediterranea* [HET]; 25º40' N - 37º40' W (surface) North Atlantic [AMT16 cruise, June 2005]; XPL
4-5. *C mediterranea* [HET]; Villefranche-sur-mer (surface) [March 2007]
6. *C mediterranea* [HET]; 30º32' N - 28'33'W Azores (surface) [June 2008]; XPL
7. *C binodata*, dispersed heterococcoliths
8. *C binodata* [HET]; 22º52' S - 24º59' W (surface) South Atlantic [AMT16 cruise, June 2005]; XPL

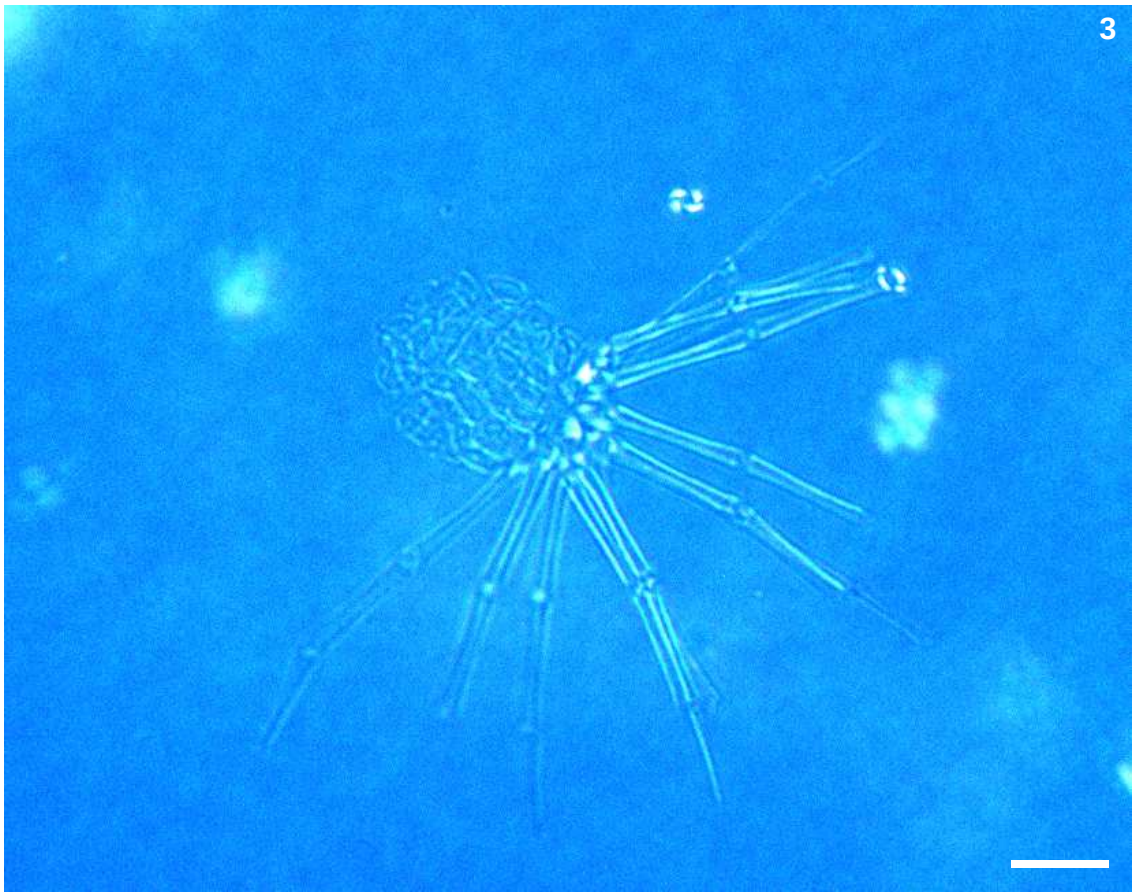


Syracosphaeraceae

Plate 32. *Michaelasarsia* Gran in Murray & Hjort 1912 emend. Manton et al. 1984 (=Halopappus Lohmann 1912)
Scale bars = 5µm
1. *M elegans* Gran 1912 emend. Manton et al. 1984 [HET] (Plankton*Net; Authors: Markus Geisen; Copyright: Jeremy Young, NHM); SEM
2. *M adriaticus* (Schiller 1914) Manton et al. 1984 [*Halopappus*] [HET] (Plankton*Net; Authors: Markus Geisen, Ian Probert and Matsunobo Kawachi; Copyright: Jeremy Young, NHM); SEM
3. *M elegans* [HET]; 31º04' S - 10º30' E (50m) South Atlantic [AMT16 cruise, May 2005]; XPL

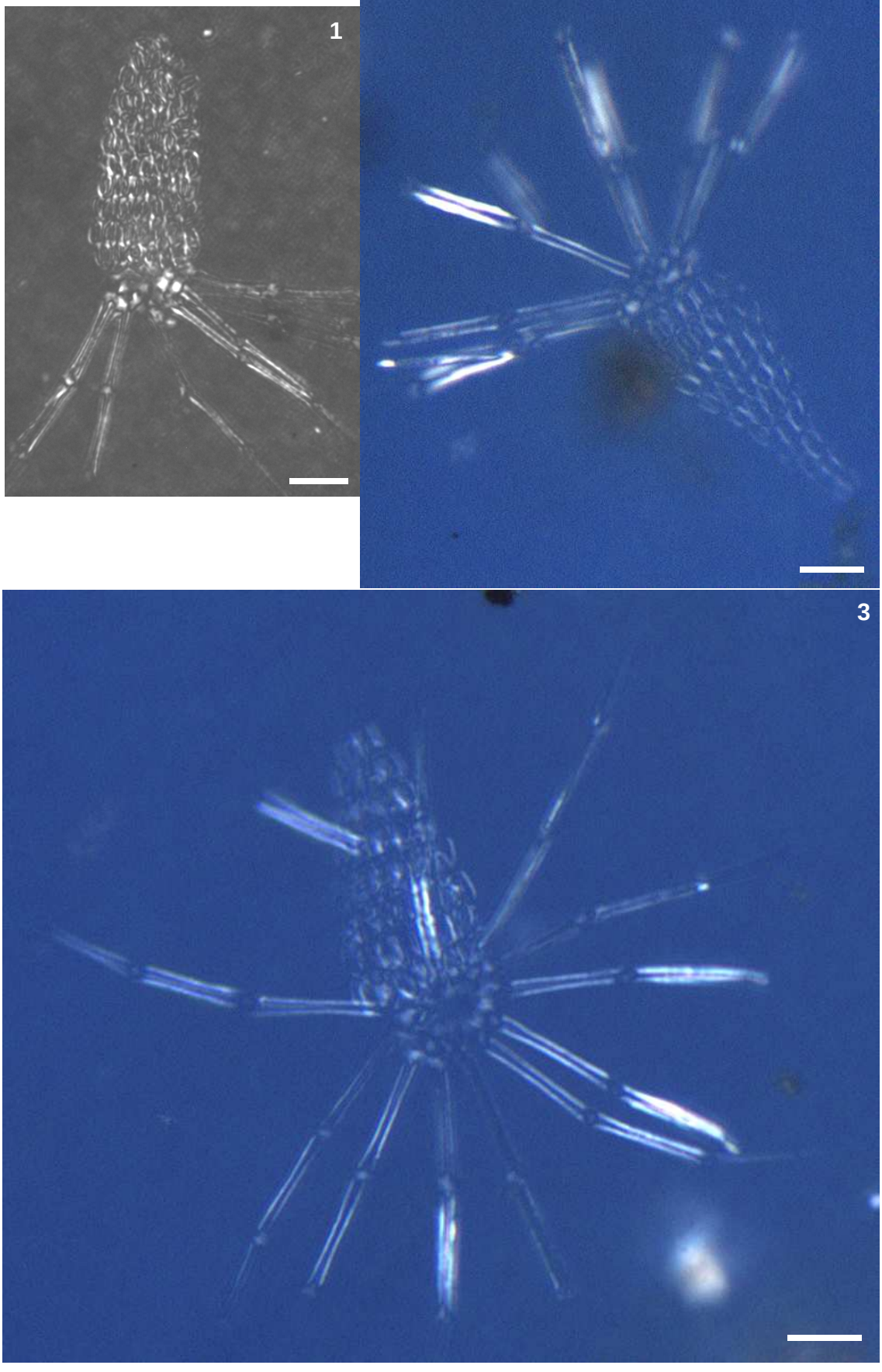






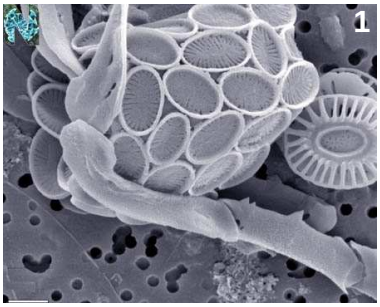
Syracosphaeraceae

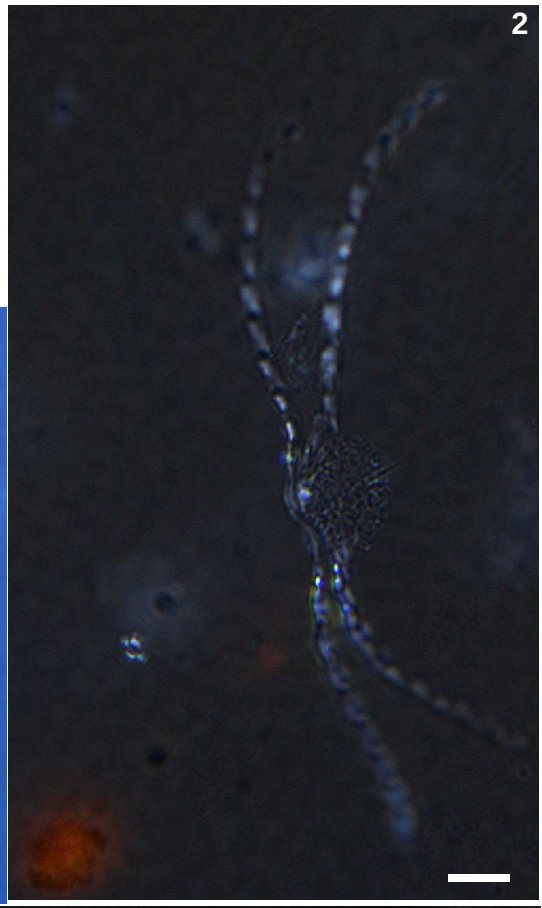
Plate 33. *Michaelasarsia* Gran in Murray & Hjort 1912 emend. Manton et al. 1984 (=Halopappus Lohmann 1912)
Scale bars = 5µm
1 and 3. *M adriaticus* [HET]; 26º31’ S - 17º13’ W (surface) South Atlantic [AMT16 cruise, May 2005]; XPL
2. *M adriaticus* [HET]; 34°04'N - 140°02'E (10m) Japan [Tansei Maru cruise, May 2006]; XPL

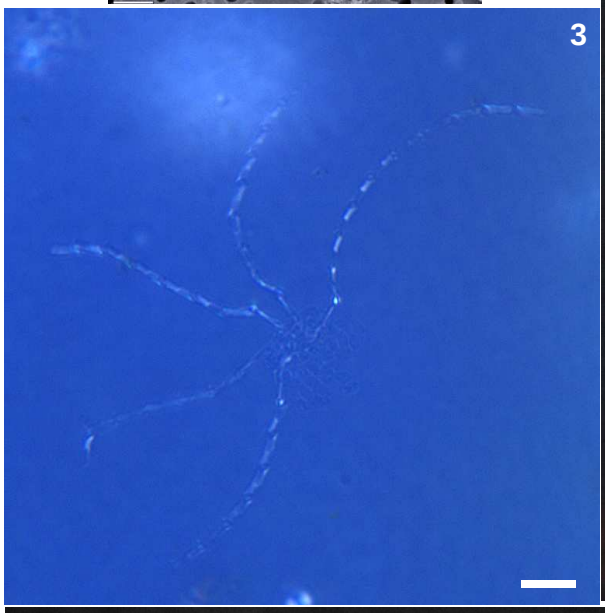


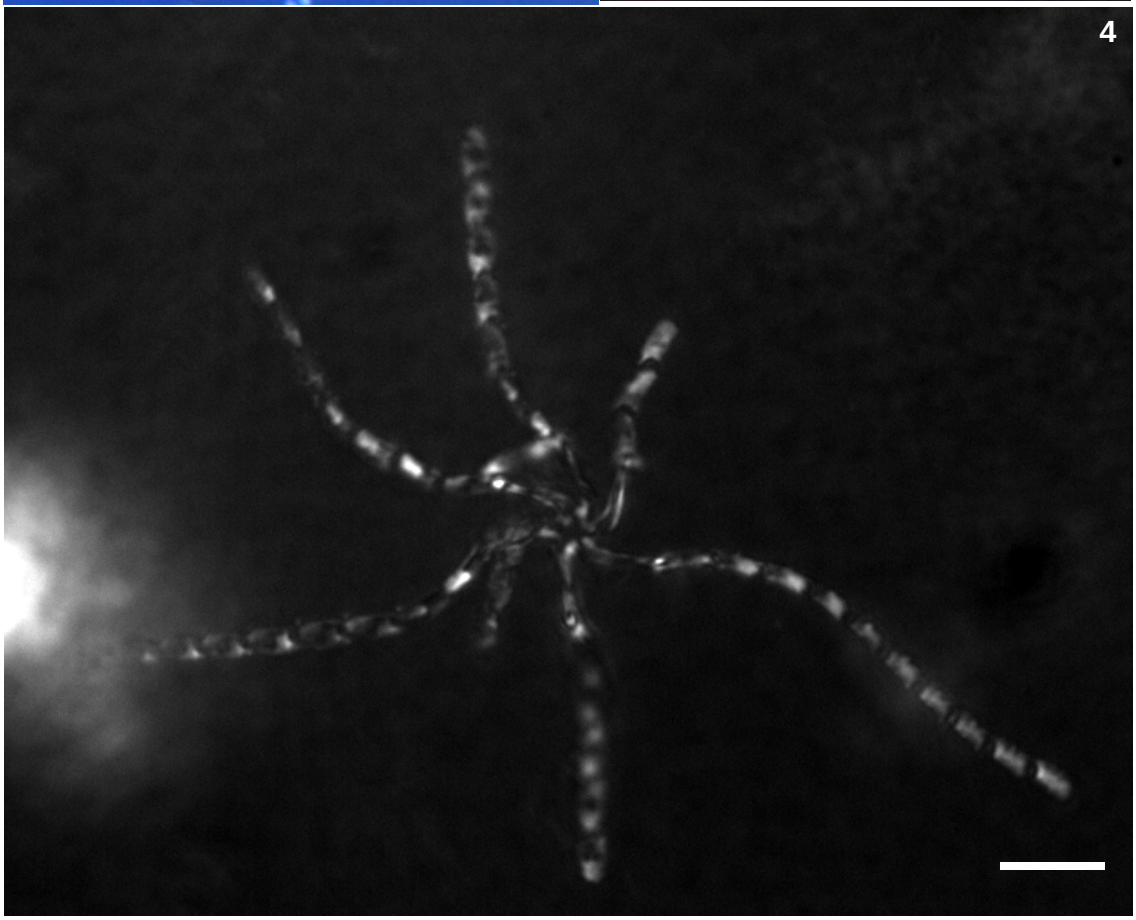
Syracosphaeraceae

Plate 34. *Ophiaster* Gran 1912 emend Manton & Oates 1983
Scale bars = 5µm
1. *Ophiaster formosus* [HET] Gran 1912 emend Manton & Oates 1983 (Plankton*Net; Authors: Markus Geisen; Copyright: Jeremy Young, NHM); SEM
2-3. *Ophiaster* sp. [HET]; 31º49’ S - 16º28’ E (96m) South Atlantic [AMT16 cruise, May 2005]; XPL
4. *Ophiaster* sp. [HET]; 20º11’ S - 24º59’ W (surface) South Atlantic [AMT16 cruise, june 2005]; XPL



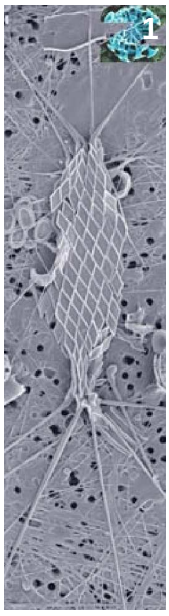


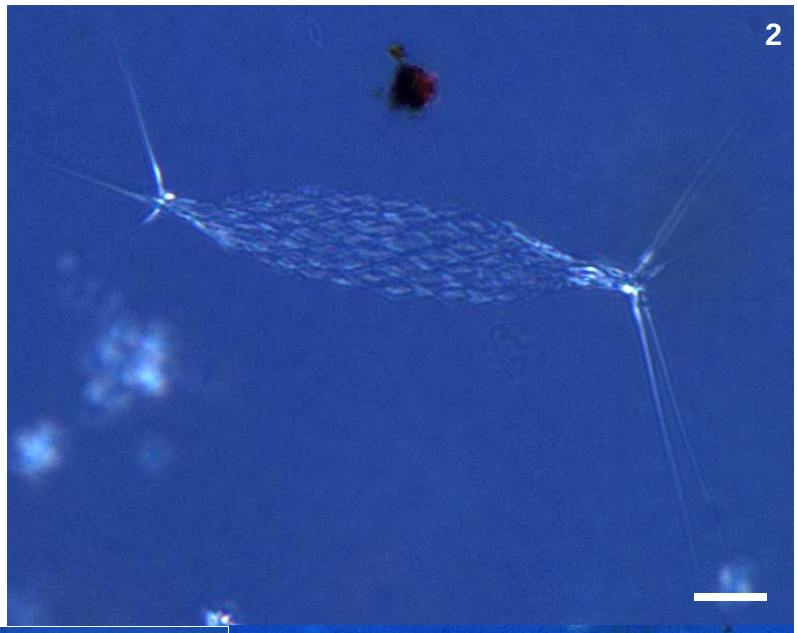


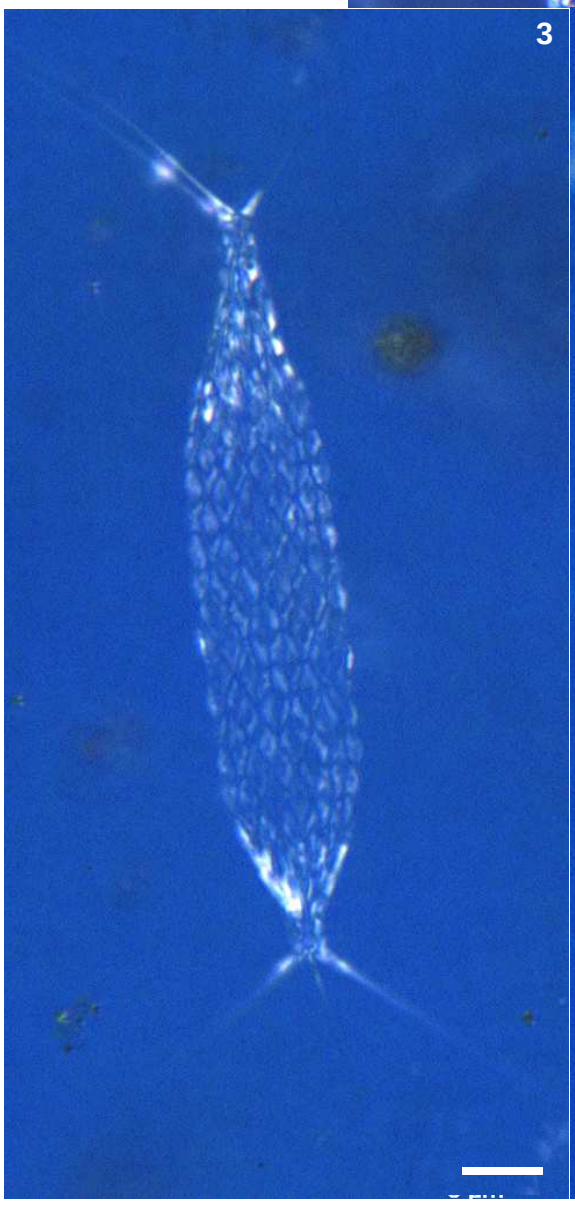


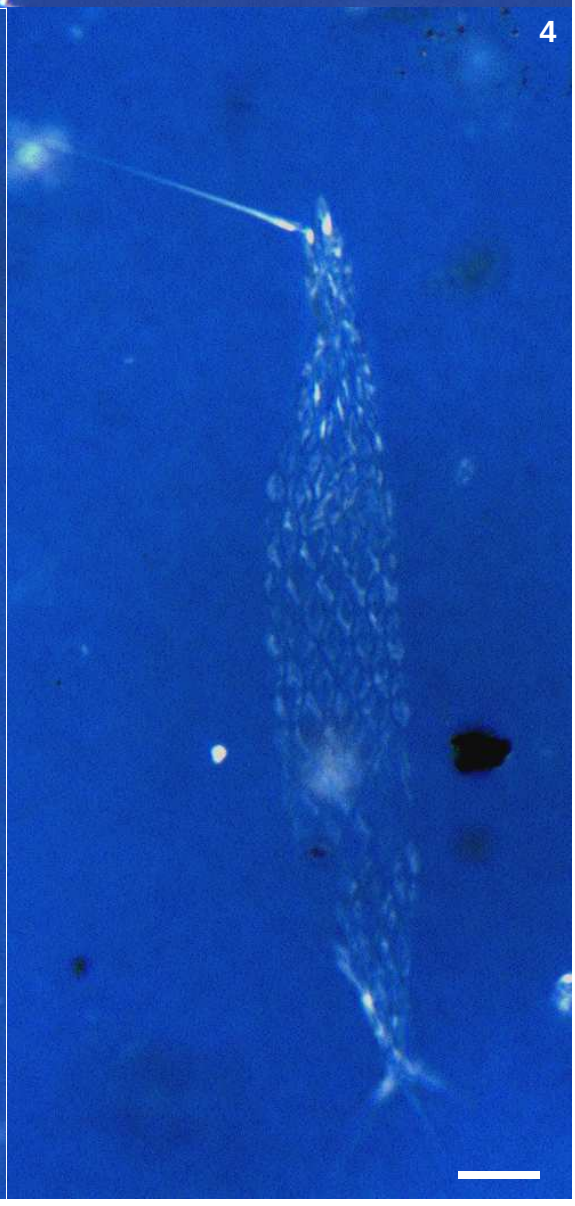
Calciosoleniaceae

Plate 35. *Calciosolenia* Gran 1912 emend.
Scale bars = 5µm
1. *C murray* [HET] Gran 1912 (Plankton*Net; Authors: Markus Geisen; Copyright: Jeremy Young, NHM); SEM
2-4. *C murray* [HET]; 26º31’ S - 17º13’ W, 38º18’ N – 30º03’ W (surface) South/North Atlantic [AMT16 cruise, May/June 2005]; XPL



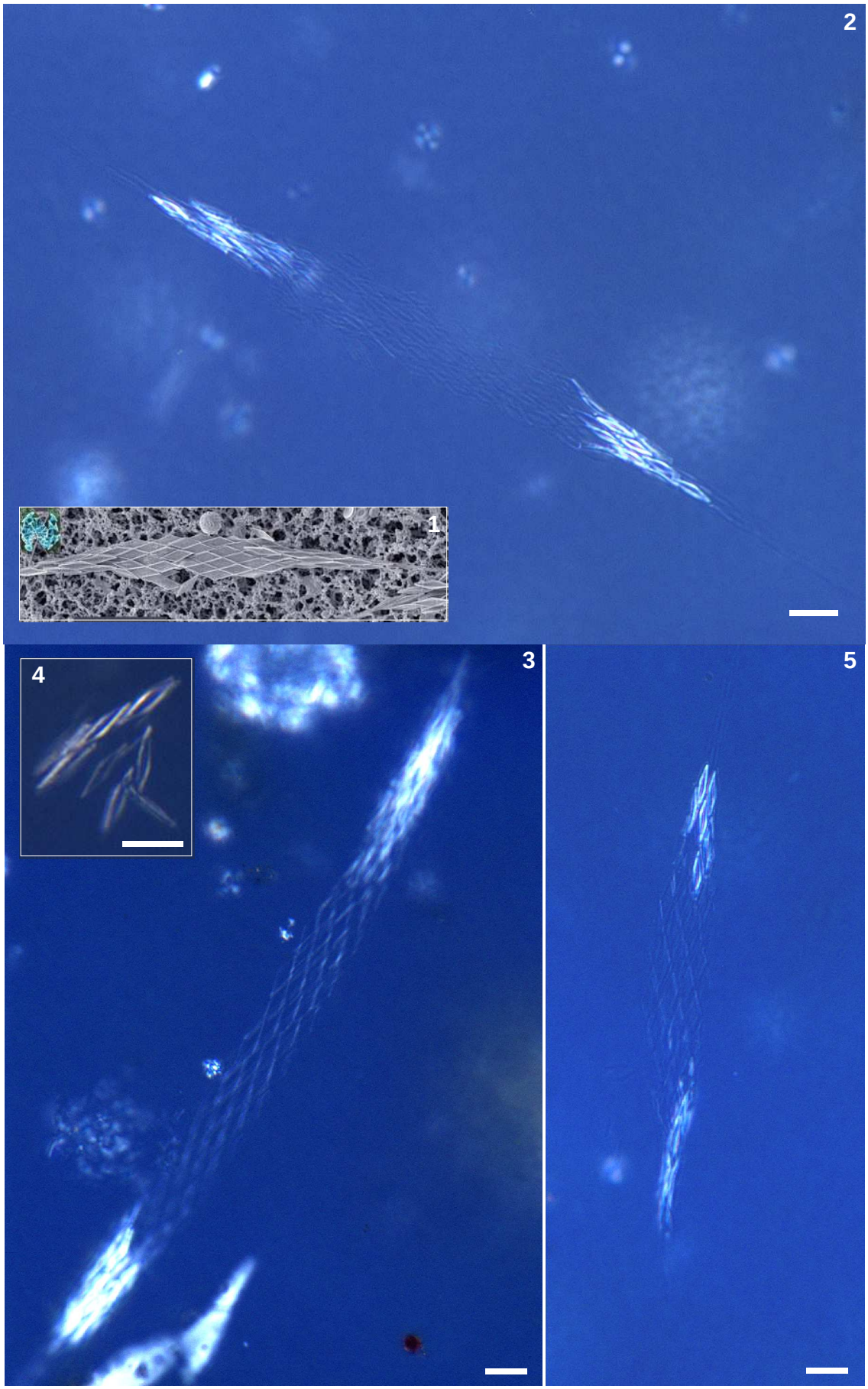






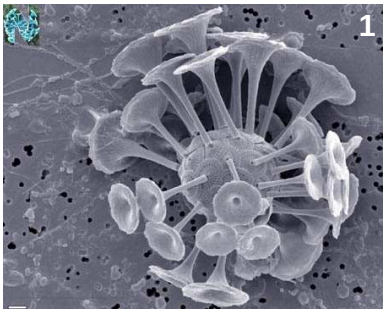
Calciosoleniaceae

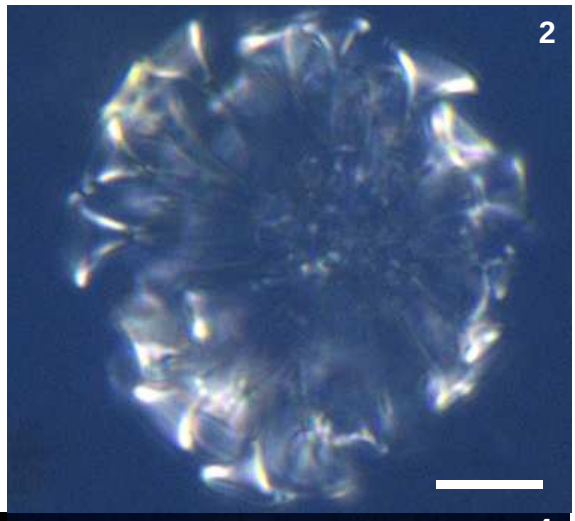
Plate 36. *Calciosolenia* Gran 1912 emend.
Scale bars = 5µm
1. *C brasiliensis* [HET] (Lohmann 1902) Young n. comb. [*Cylindrotheca*] (Plankton*Net; Authors: Markus Geisen; Copyright: Jeremy Young, NHM); SEM
2 and 4. *C brasiliensis* [HET]; 31º49’ S - 16º28’ E (96m) South Atlantic [AMT16 cruise, May 2005]; XPL
3. *C brasiliensis* [HET]; 26º31’ S - 17º13’ W (surface) South Atlantic [AMT16 cruise, May 2005]; XPL
5. *C brasiliensis* [HET], dispersed heterococcoliths; 20º11’ S - 24º59’ W (surface) South Atlantic [AMT16 cruise, June 2005]; XPL

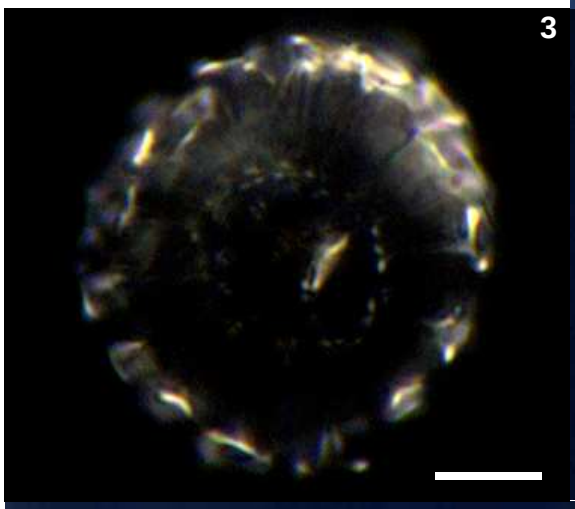

2
1
3
4
5

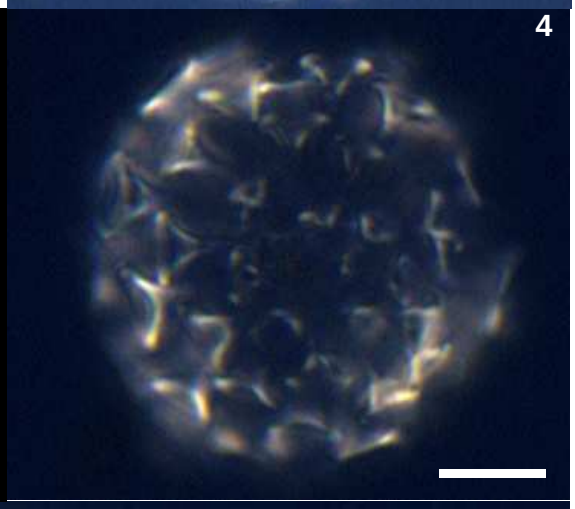
Rabdosphaeraceae

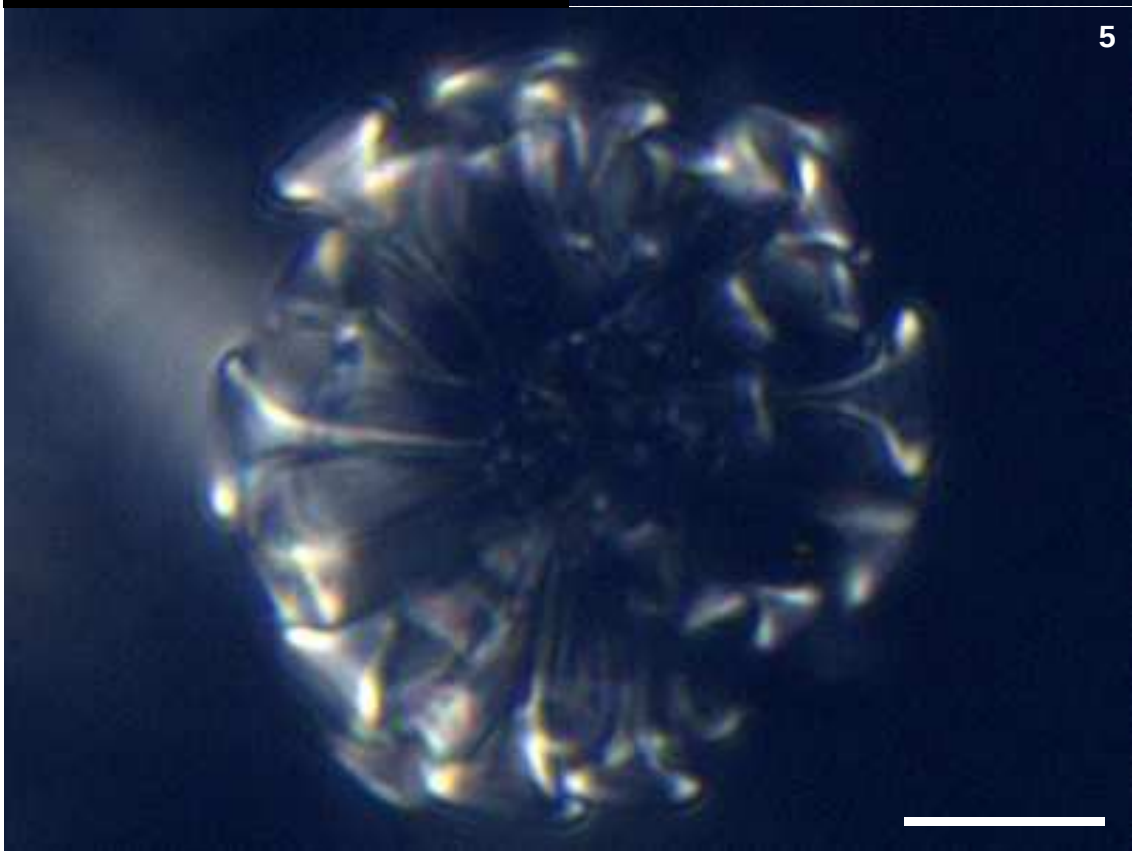
Plate 37. *Discosphaera* Haeckel 1894
Scale bars = 5µm
1. *D. tubifera* [HET] (Murray & Blackman 1898) Ostenfeld 1900 [Rhabdosphaera]
(Plankton*Net; Authors: Markus Geisen; Copyright: Jeremy Young, NHM); SEM
2. *D. Tubifera* [HET]; 26º31’ S - 17º13’ W (surface) South Atlantic [AMT16 cruise, May 2005]; XPL
3-4. *D. tubífera* [HET]; 20º11’ S - 24º59’ W (surface) South Atlantic [AMT16 cruise, June 2005]; XPL
5. *D. tubífera* [HET]; 25º40’ N – 37º40’ W (surface) North Atlantic [AMT16 cruise, June 2005]; XPL


1


2

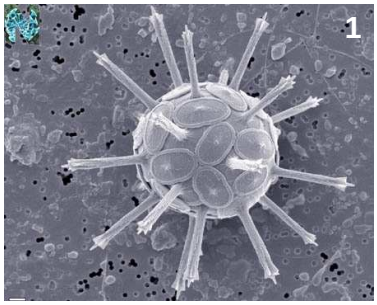

3

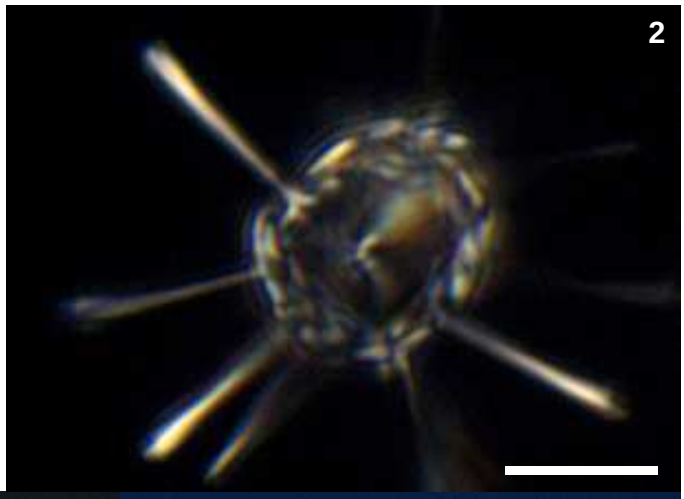

4

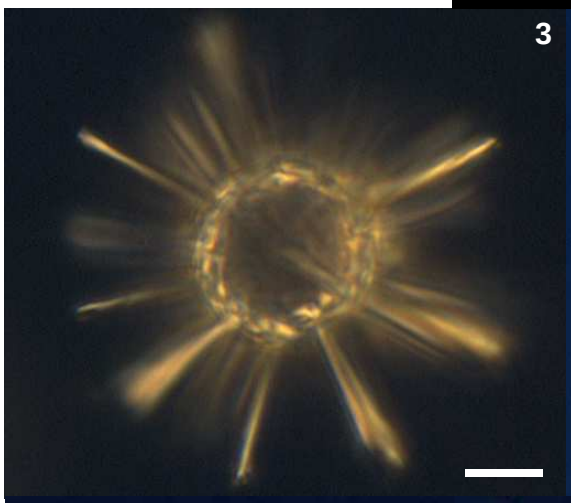

5

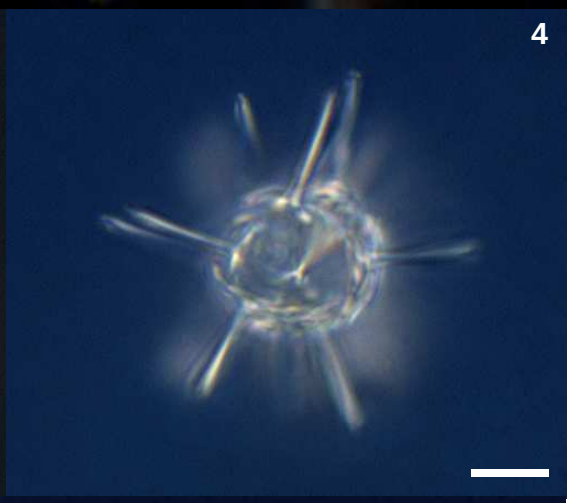
Rabdosphaeraceae

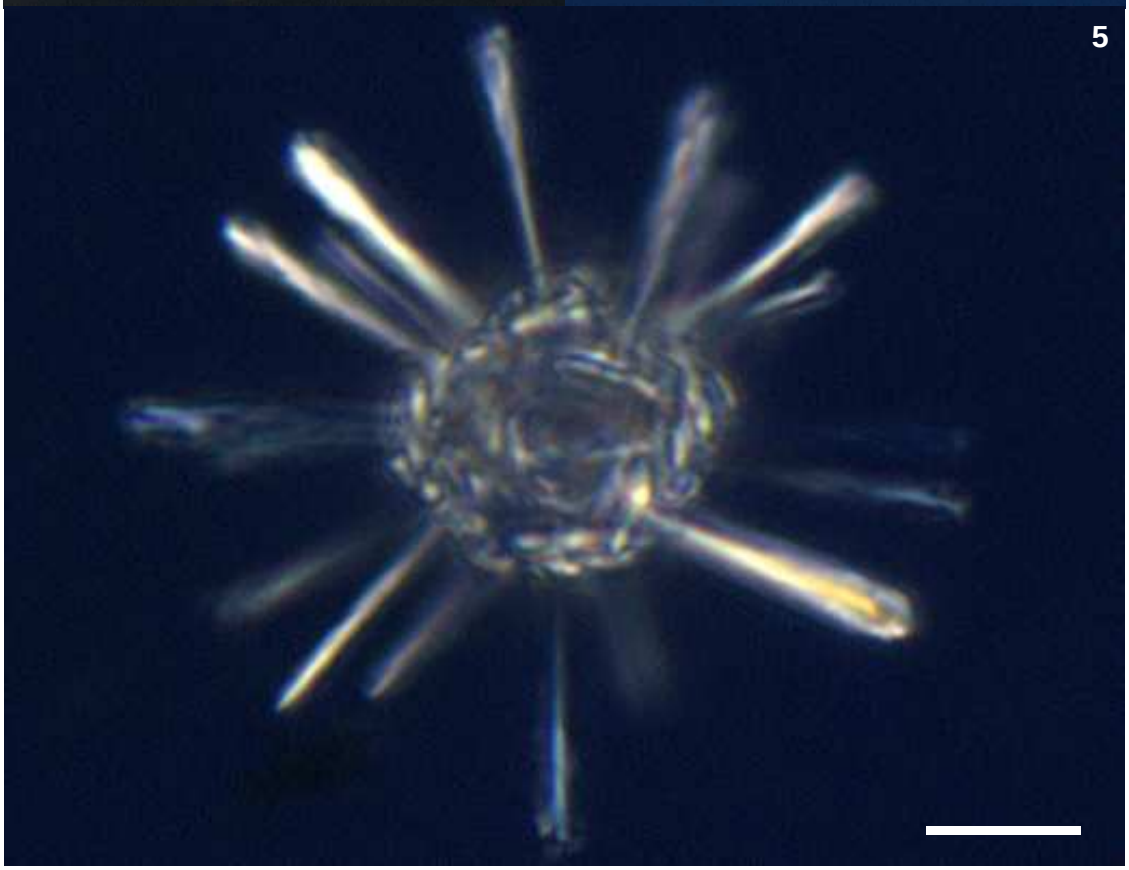
Plate 38. *Rhabdosphaera* Haeckel 1894
Scale bars = 5µm
1. *R. Clavigera* [HET] Murray & Blackman 1898 (Plankton*Net; Authors: Markus Geisen; Copyright: Jeremy Young, NHM); SEM
2-3. *R. Clavigera* [HET]; 20º11’ S - 24º59’ W (surface) South Atlantic [AMT16 cruise, June 2005]; XPL
4-5. *R. Clavigera* [HET]; 26º31’ S - 17º13’ W (surface) South Atlantic [AMT16 cruise, May 2005]; XPL


1


2

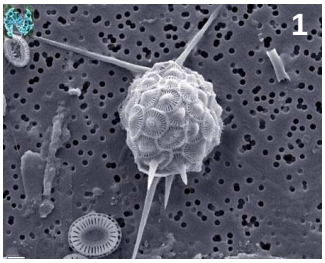

3

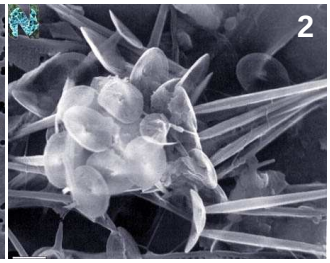

4

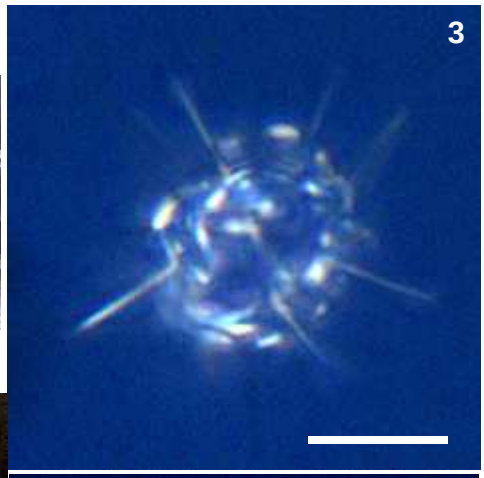

5

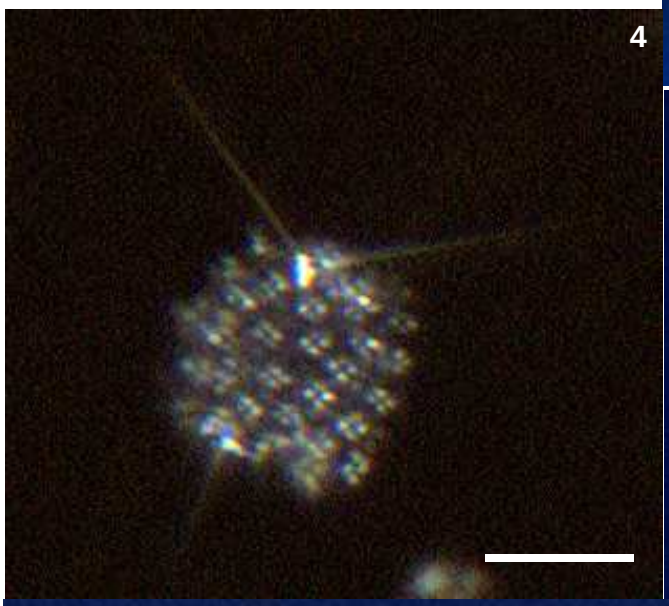
Rabdosphaeraceae

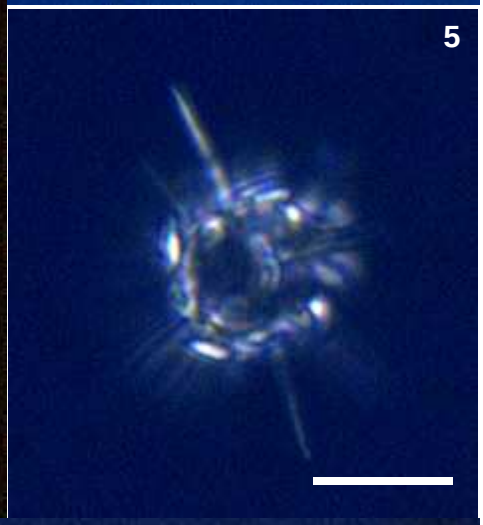
Plate 39. *Acanthoica* Lohmann 1903 emend. Schiller 1913 and Kleijne 1992 & *Palusphaera* Lecal 1965 emend. Norris 1984
Scale bars = 5µm
1. *Acanthoica quattrospina* Lohmann 1903 [HET] (Plankton*Net; Authors: Jeremy Young; Copyright: Jeremy Young, NHM); SEM
2. *Palusphaera* sp. 1 Cros Fortuño 2002 [HET], collapsed coccosphere (Plankton*Net; Authors: Annelies Kleijne; Copyright: Annelies Kleijne, VU Amsterdam); SEM
3-4. *Palusphaera* sp. [HET]; 20º11' S - 24º59' W (surface) South Atlantic [AMT16 cruise, June 2005]; XPL
5-6. *Acanthoica* sp. [HET]; 22º52' S – 24º59' W (surface) South Atlantic and 36º27'N – 36º55' W (67m) North Atlantic [AMT16 cruise, May/June 2005]; XPL

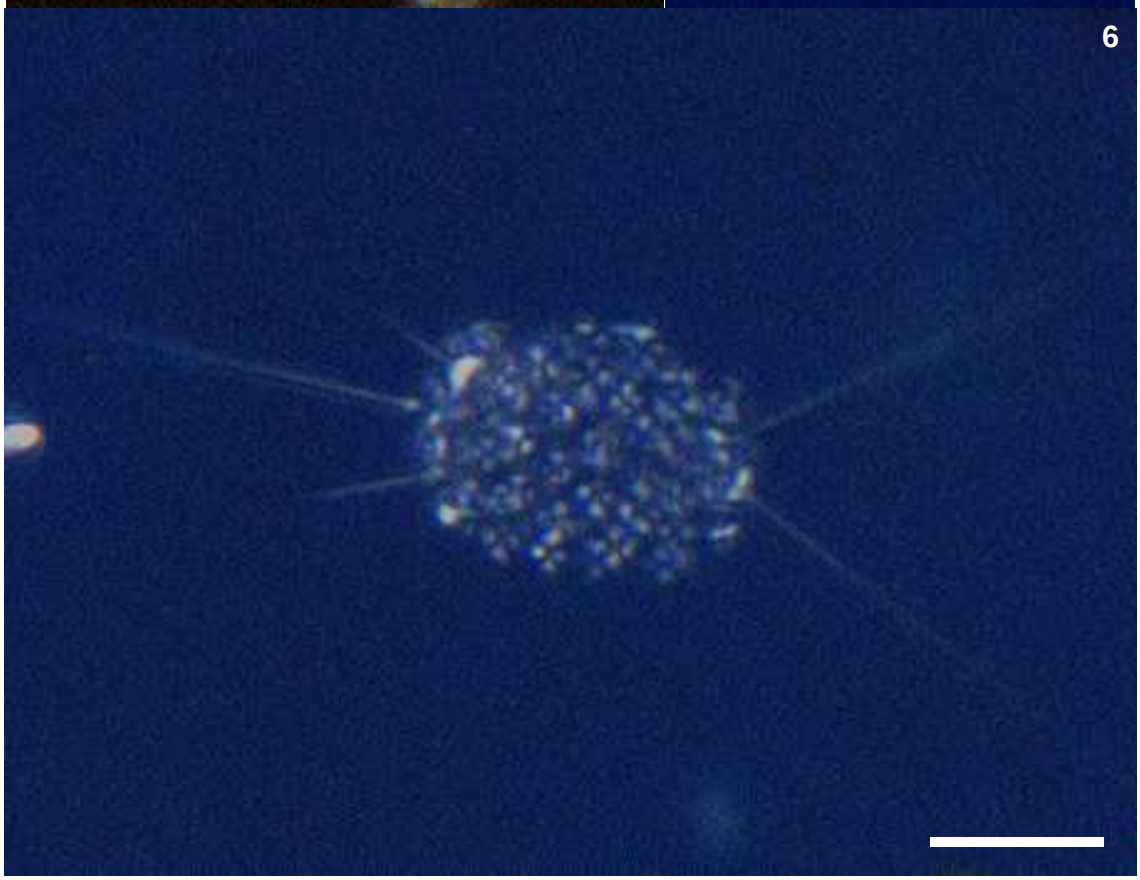

1


2


3

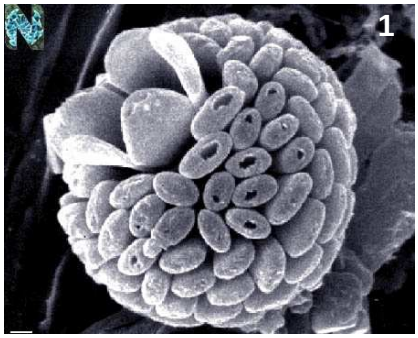

4

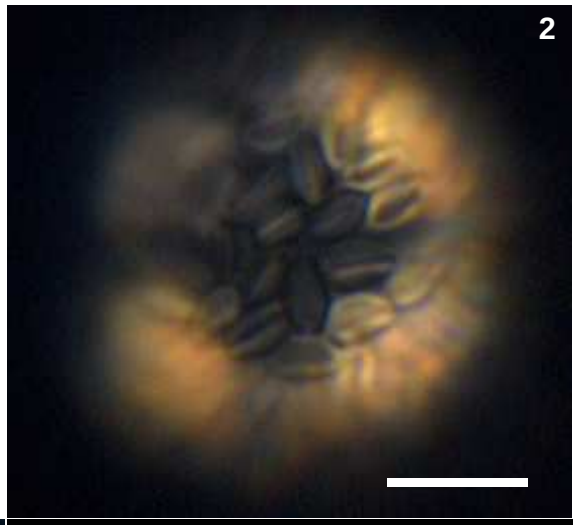

5

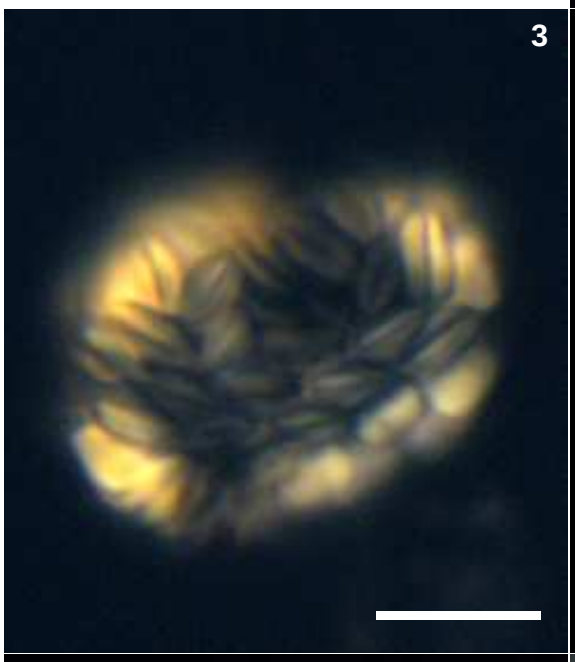

6

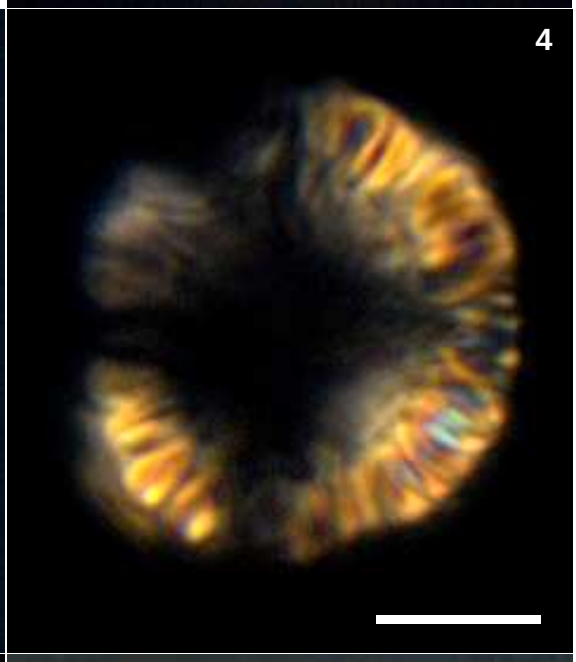
Rabdosphaeraceae

Plate 40. *Algirosphaera* Schlauder 1945 emend Norris 1984
Scale bars = 5µm
1. *A robusta* [HET] (Lohmann 1902) Norris 1984 [Syracosphaera] (Plankton*Net; Authors: Vita Pariente; Copyright: Vita Pariente, Texas A&M); SEM
2,4 and 5. *A robusta* [HET]; 20º11' S - 24º59' W (surface) South Atlantic [AMT16 cruise, June 2005]; XPL
3 and 6. *A robusta* [HET]; 01º37' S – 24º59' W (surface) South Atlantic [AMT16 cruise, June 2005]; XPL


1


2


3

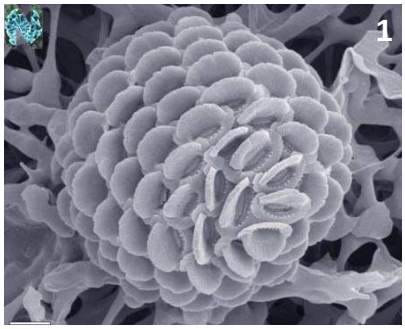

4

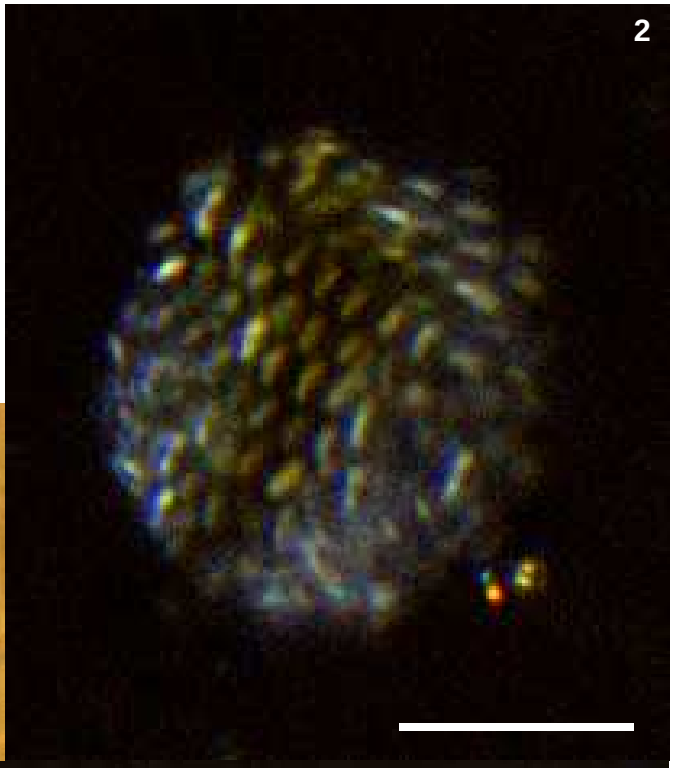

5

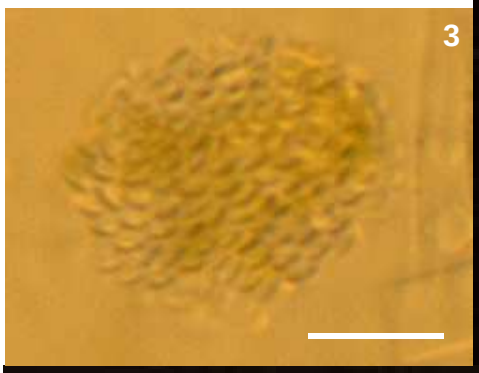

6

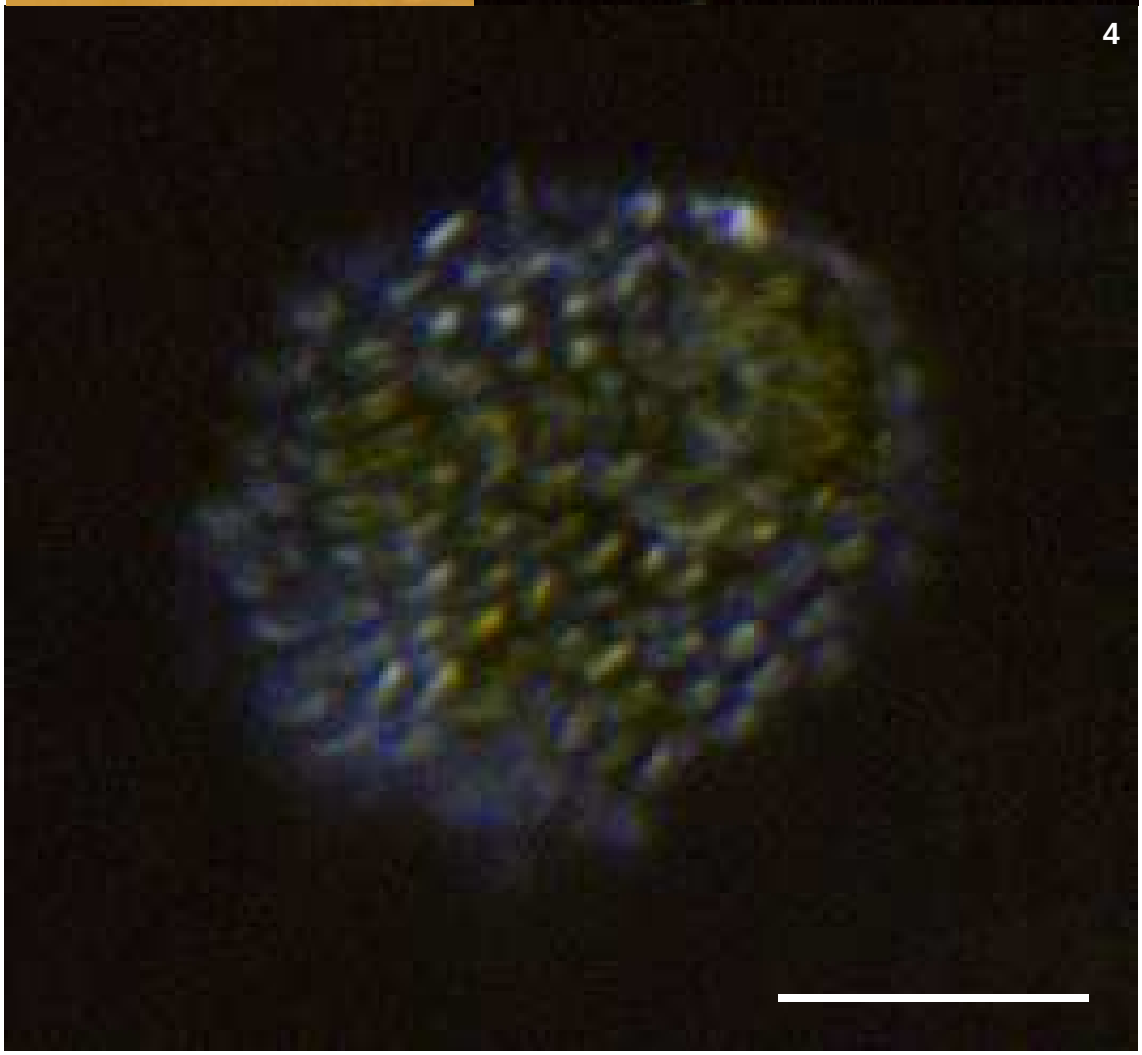
Incertis sedis

Plate 41. *Alisphaera* Heimdal 1973
Scale bars = 5µm
1. *A ordinata* (Kampter 1941) Heimdal 1973 (Plankton*Net; Authors: Markus Geisen; Copyright: Jeremy Young, NHM); SEM
2-4. *Alisphaera* sp. [HET]; 30º32’ N - 28’33’W Azores Faial (30m) [May 2008]; XPL


1

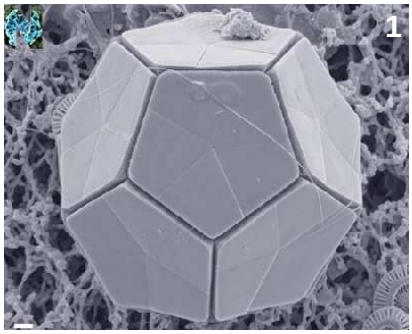

2

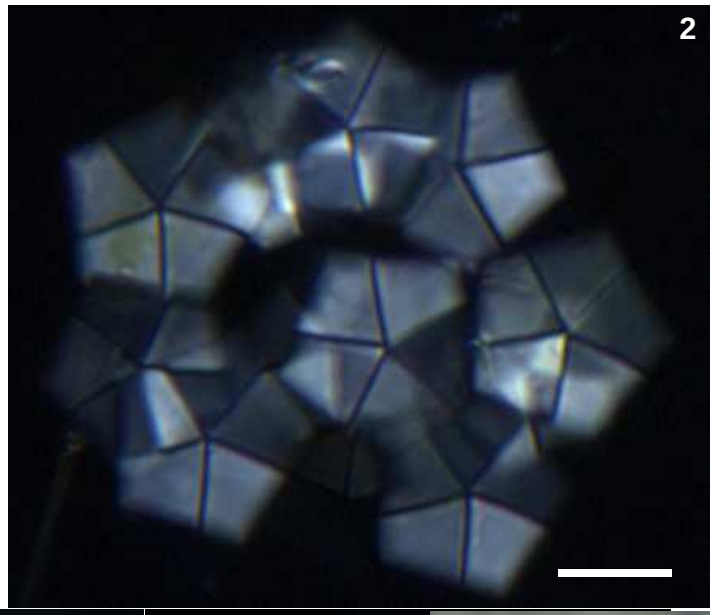

3


4

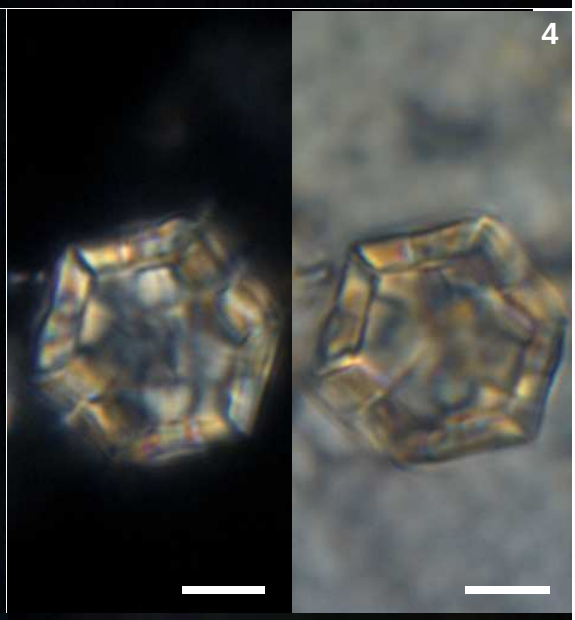
Incertis sedis

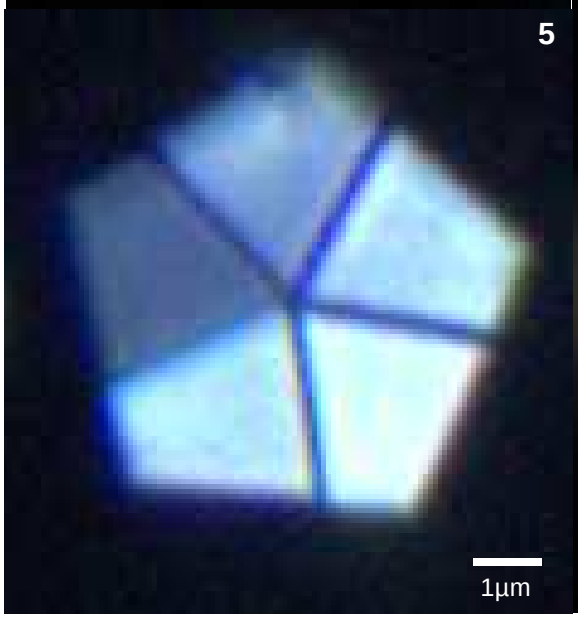
Plate 42. *Braarudosphaera* Deflandre 1947 [*Pontosphaera*]
Scale bars = 5µm
1. *B bigelowii* (Gran & Braarud 1935) Deflandre 1947 [*Pontosphaera*]
(Plankton*Net; Authors: Jeremy Young; Copyright: Jeremy Young, NHM); SEM
2. *B bigelowii* [HET], desintegrated cell; 30º32’ N - 28’33’W Azores, Faial [June 2008]; XPL
3-6. *B bigelowii* [HET], whole cells and isolated nannolith; 30º32’ N - 28’33’W Azores, Faial [July 2008; XPL

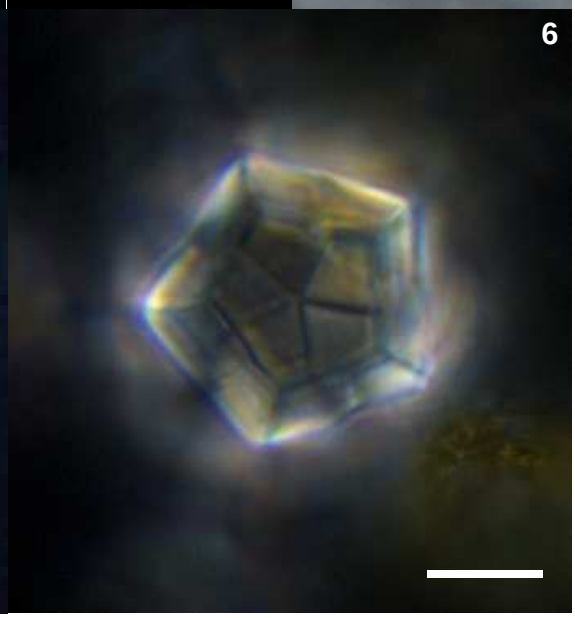

1


2


3

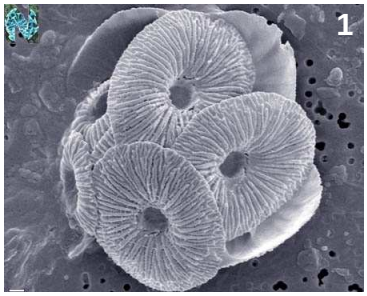

4

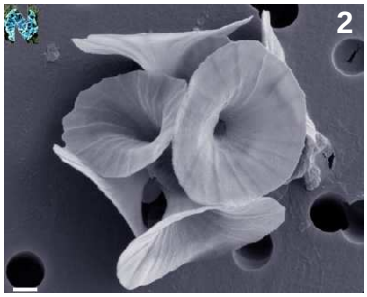

5
1µm

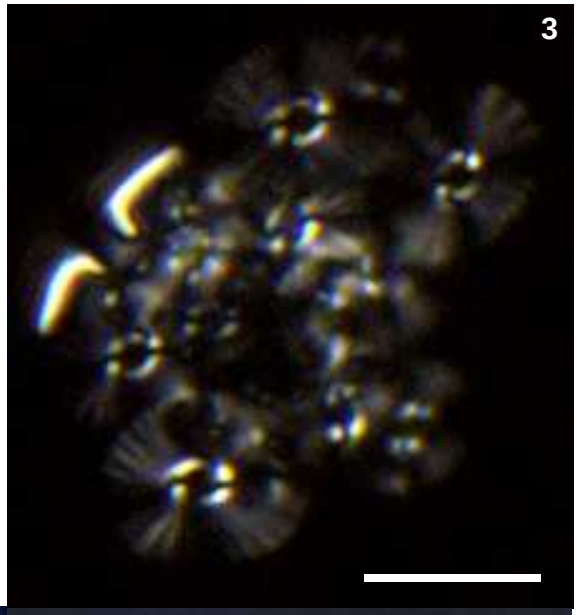

6

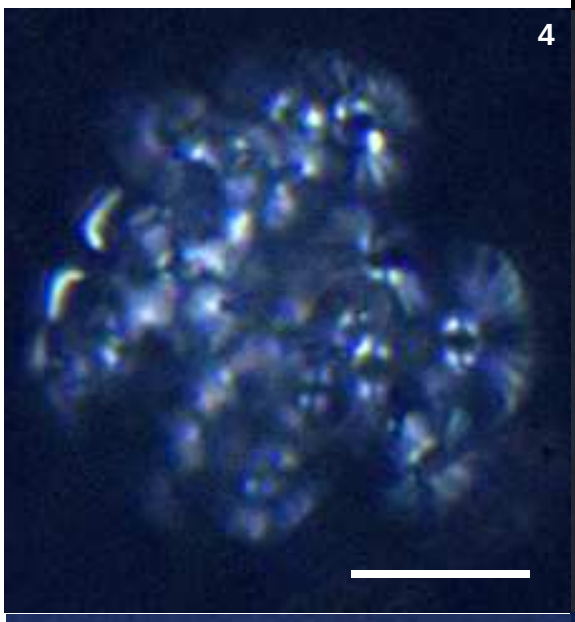
Incertis sedis

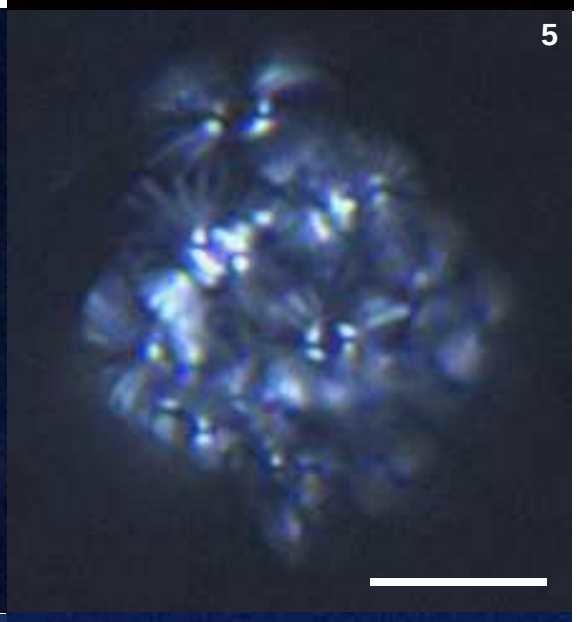
Plate 43. *Umbelosphaera* Paasche *in* Markali & Paasche 1955
Scale bars = 5µm
1. *U tenuis* type IV (Kampter 1937) Paasche *in* Markali & Paasche 1955 [*Coccolithus*] (Plankton*Net; Authors: Markus Geisen; Copyright: Jeremy Young, NHM); SEM
2. *U irregularis* Paasche *in* Markali & Paasche 1955 (Plankton*Net; Authors: Jeremy Young; Copyright: Jeremy Young, NHM); SEM
3-5. *U tenuis* [HET]; 35º05' N – 41º51' W (39m) North Atlantic [AMT16 cruise, June 2005]; XPL
6-7. *U irregularis* [HET]; 22º27' S - 24º59' W (surface), 20º11 S - 24º59 W (surface) South Atlantic [AMT16 cruise, June 2005]; XPL

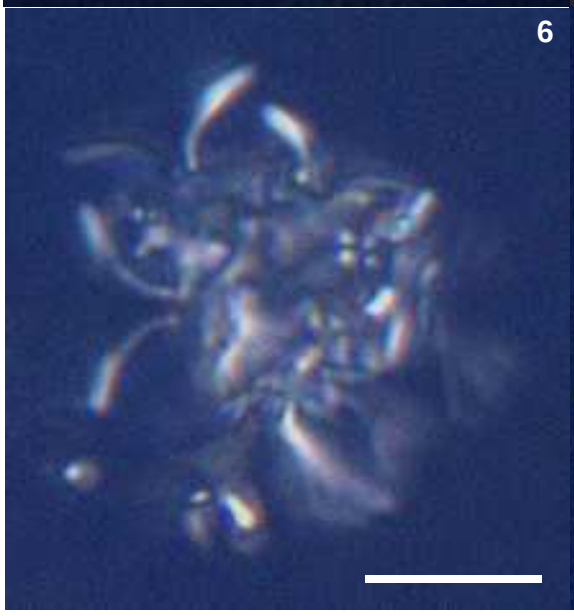


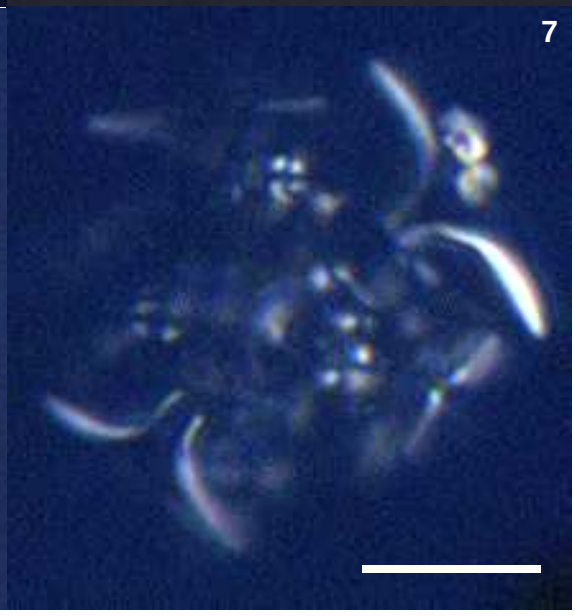






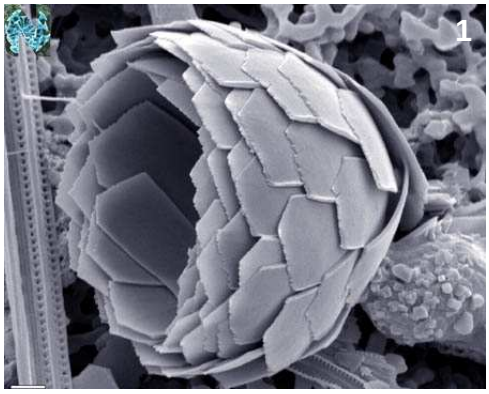


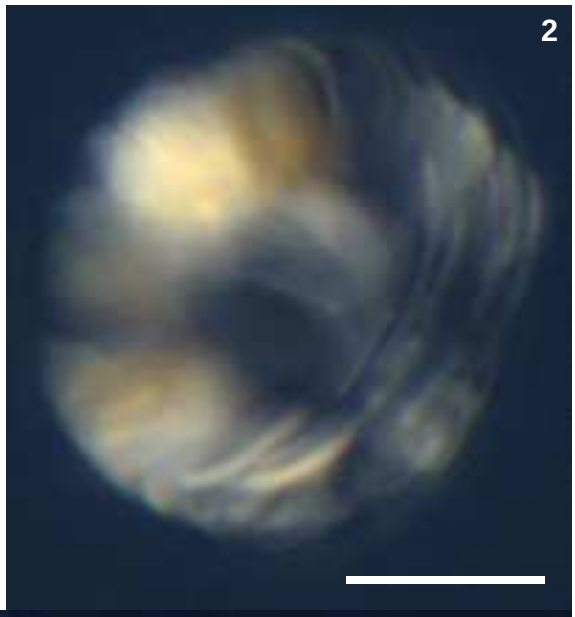


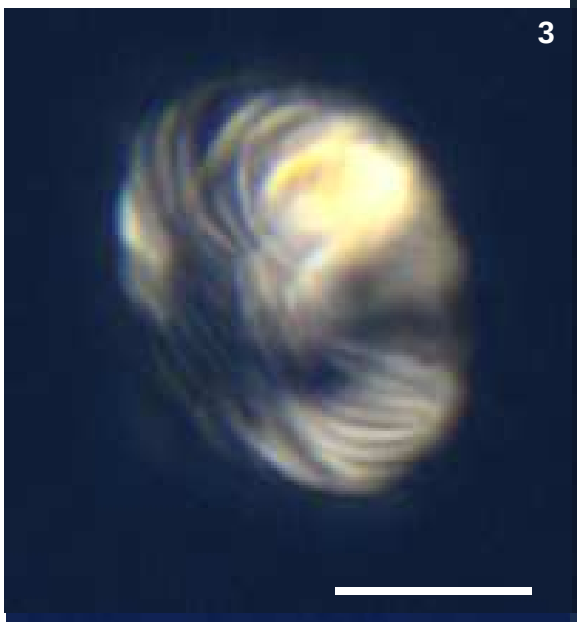


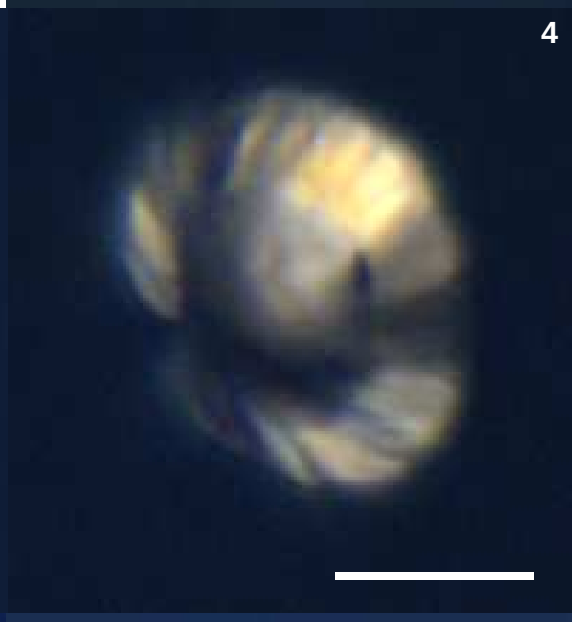
Incertis sedis

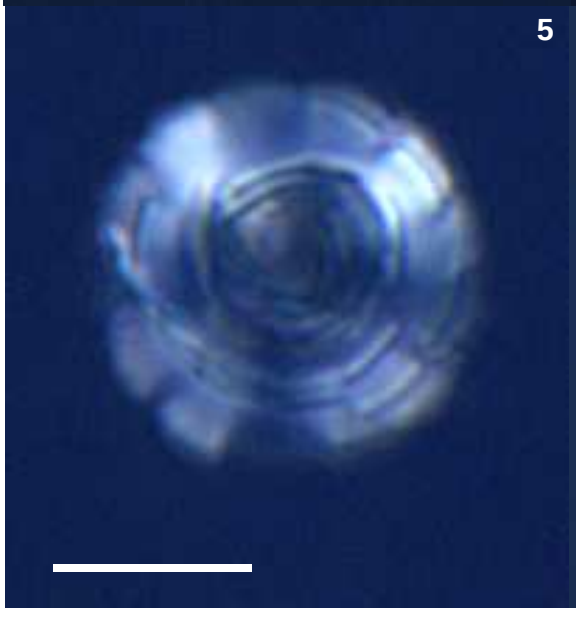
Plate 44. *Florisphaera* Okada & Honjo 1973
Scale bars = 5µm
1 *F profunda* Okada & Honjo 1973 (Plankton*Net; Authors: Vita Pariente and Jeremy Young; Copyright: Jeremy Young, NHM); SEM
2. *F profunda* [HET]; 31º58' S – 16º58' E (50m) South Atlantic [AMT16 cruise, June 2005]; XPL
3-4. *F profunda* [HET]; 20º11' S – 24º59' W (surface, night) South Atlantic [AMT16 cruise, June 2005]; XPL
5-6. *F profunda* [HET]; 22º52' S – 24º59' W (surface, night) South Atlantic [AMT16 cruise, June 2005]; XPL

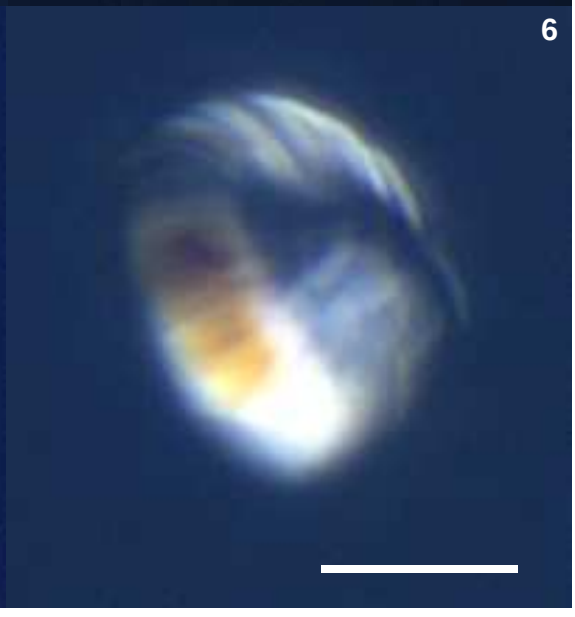

1


2


3

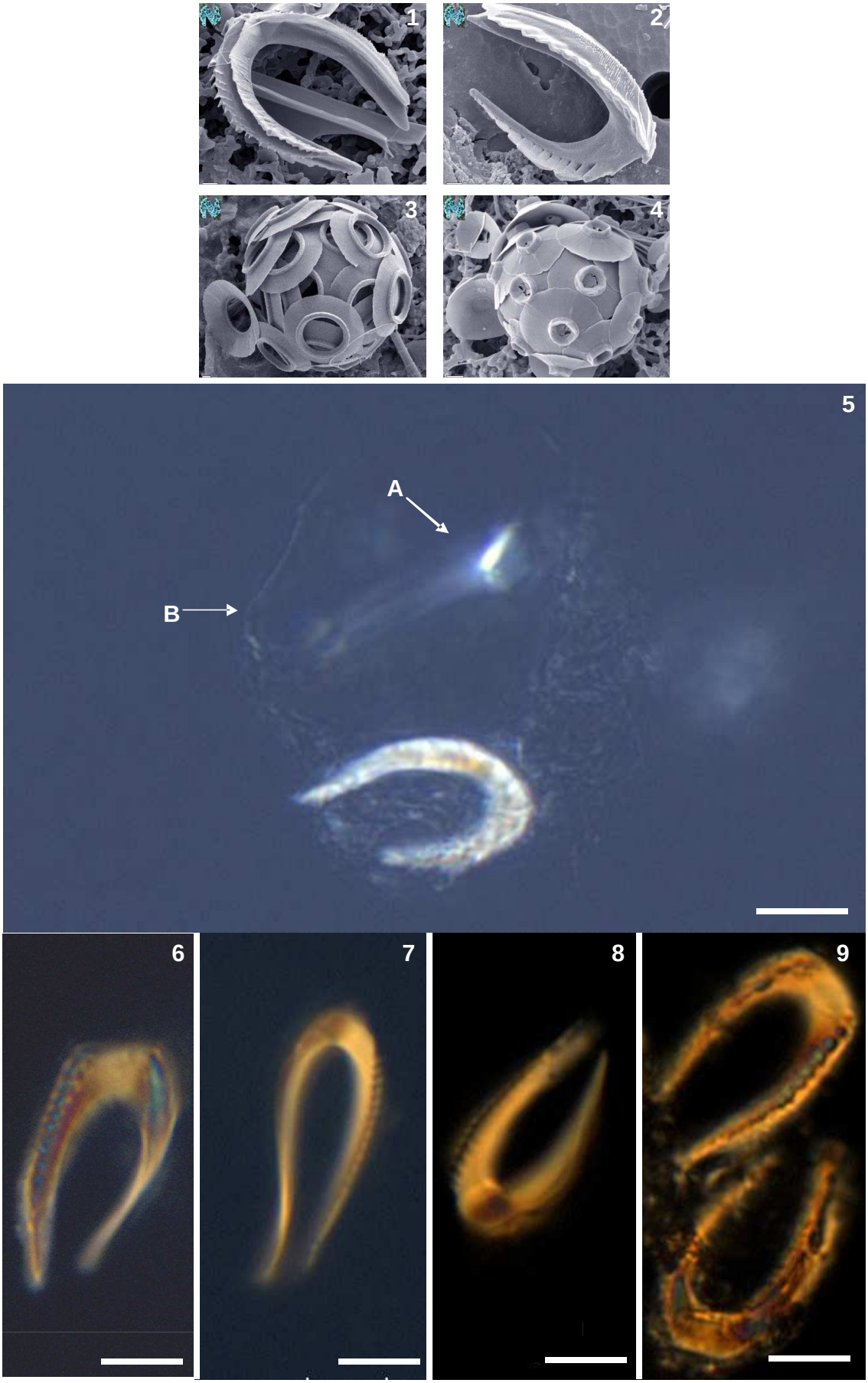

4


5


6

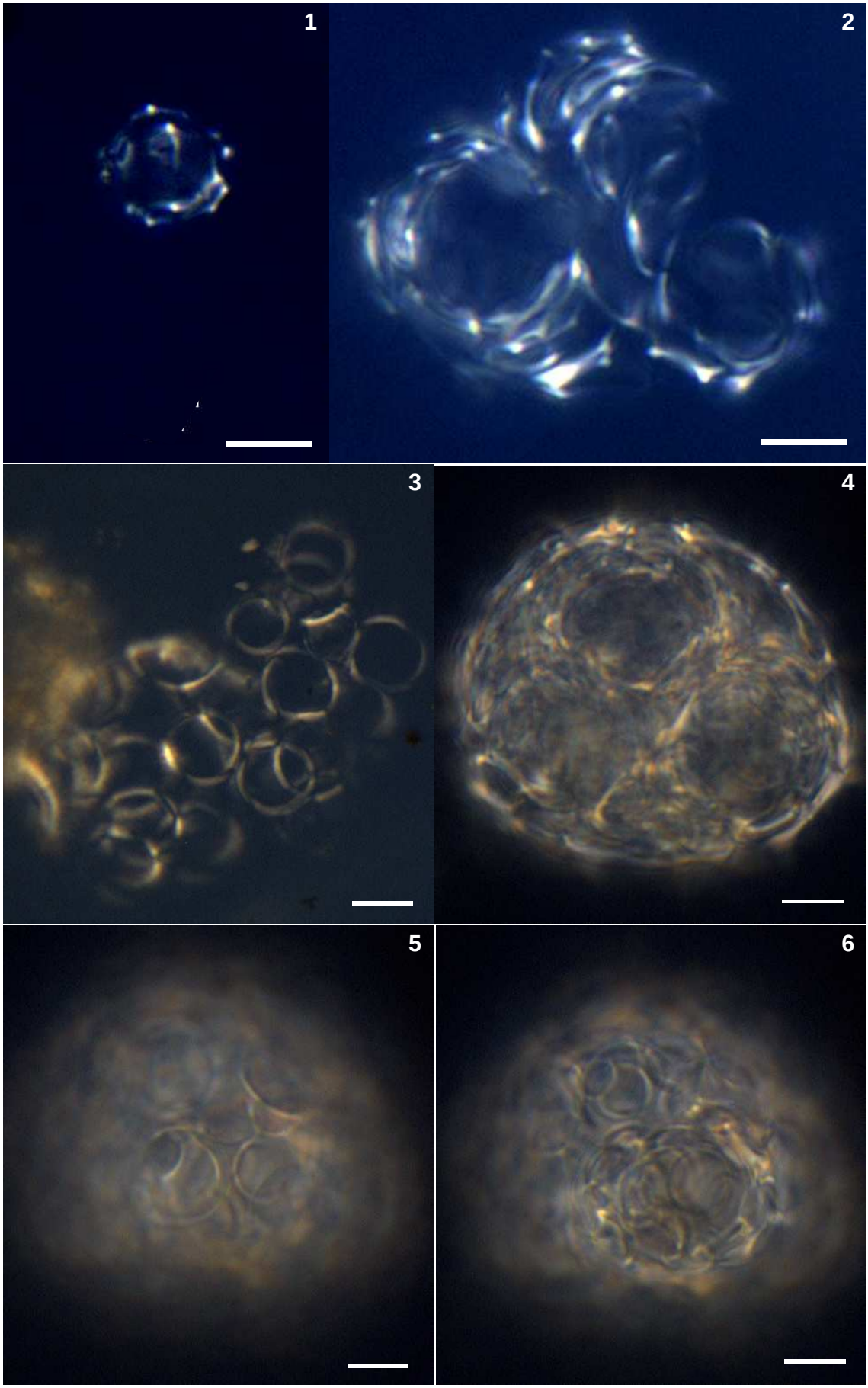
Incertis sedis

Plate 45. *Ceratolithaceae* Norris 1965
Scale bars = 5µm
1. *C cristatus* Kampter 1950 [CER] cristatus type (base view), according to Young et al. 2003 it is likely that the ceratolithus stage (CER) is equivalent to the holococcolith stage in other taxa, and so haploid (Plankton*Net; Authors: Vita Pariente and Jeremy Young; Copyright: Jeremy Young, NHM); SEM
2. C cristatus Kampter 1950 [CER] cristatus type (top view) (Plankton*Net; Authors: Markus Geisen; Copyright: Jeremy Young, NHM); SEM
3. *C cristatus* [HET] coccolithomorpha type [= *Neosphaera coccolithomorpha* Lecal-Schlauder 1950] (Plankton*Net; Authors: Vita Pariente and Claire Findlay; Copyright: Jeremy Young, NHM); SEM
4. *C cristatus* [HET] nishidae type [= *Neosphaera coccolithomorpha* var. *nishidae*] (Plankton*Net; Authors: Claudia Sprengel and Jeremy Young; Copyright: Jeremy Young, NHM); SEM
5. *C cristatus* [CER], note the presence of two horse-shoe nannoliths (arrow A) placed more or less in parallel to each other, but placed in opposite sides, inside a common membrane-like structure (arrow B); 31º49' S – 10º30' E (96m) South Atlantic [AMT16 cruise, June 2005]; XPL
6. *C cristatus* [CER]; 31º04' S - 16º28' E (50m) South Atlantic [AMT16 cruise, May 2005]; XPL
7-8. *C cristatus* [CER]; 31º34' S – 09º19' E (surface) South Atlantic [AMT16 cruise, May 2005]; XPL
9. *C cristatus* [CER]; 31º58' S – 16º58' E (50m) South Atlantic [AMT16 cruise, June 2005]; XPL



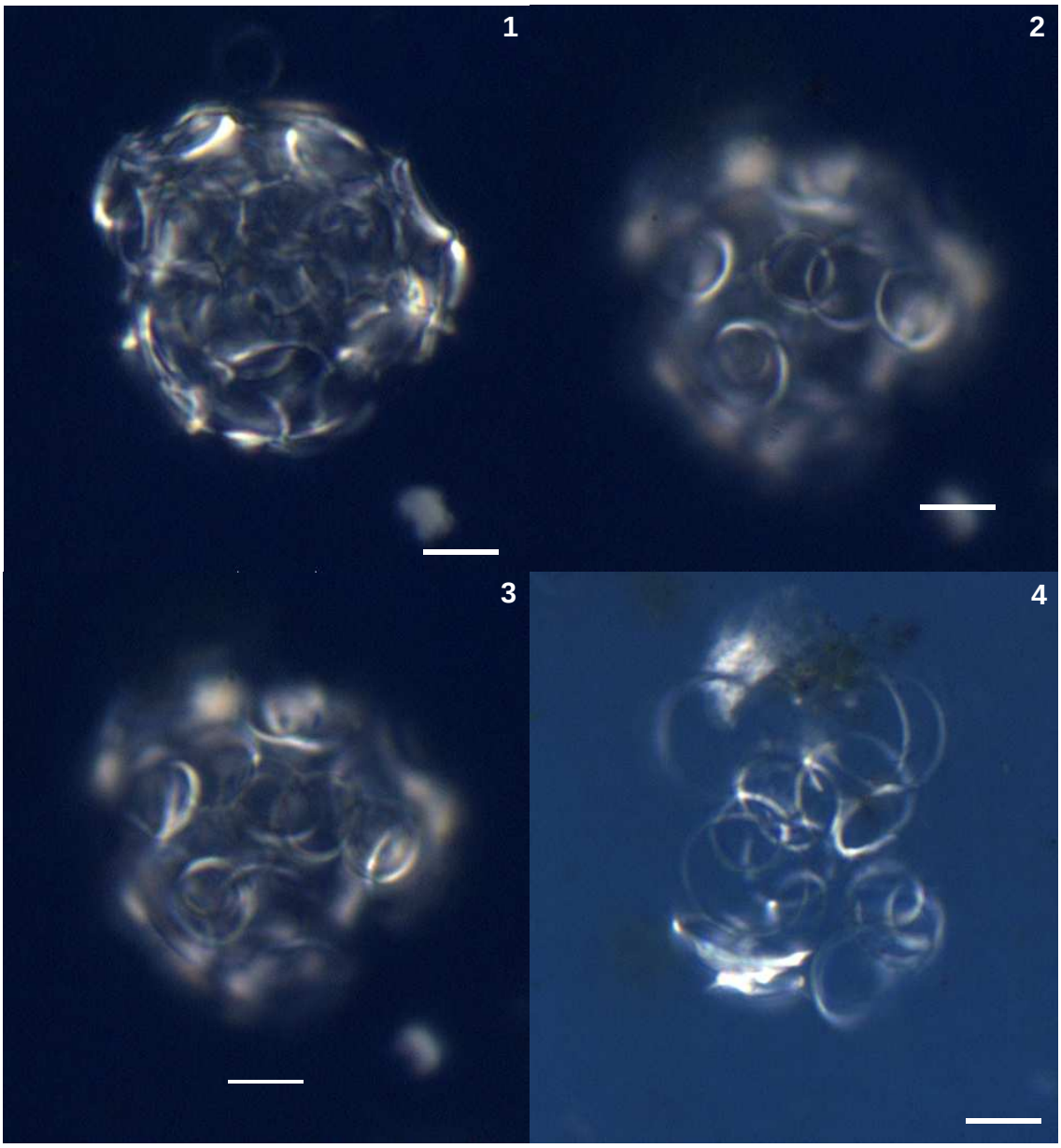
Incertis sedis

Plate 46. *Ceratolithaceae* Norris 1965
Scale bars = 5µm
1. *C cristatus* [HET] – probably nishidae type
2. *C cristatus* [HET] nishidae type; 31º04 S - 16º28 E (50m) South Atlantic [AMT16 cruise, May 2005]; XPL
3. *C cristatus* [HET] coccolithomorpha type, dispersed coccoliths; 31º58 S - 16º58 E (10m) South Atlantic [AMT16 cruise, May 2005]; XPL
4-6. *C cristatus* [HET] coccolithomorpha type (same coccosphere viewed at different phocal planes); 31º04 S - 16º28 E (50m) South Atlantic [AMT16 cruise, May 2005]; XPL



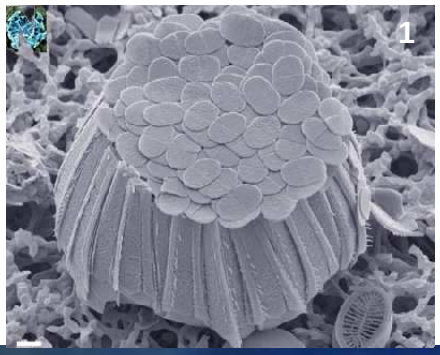
Incertis sedis

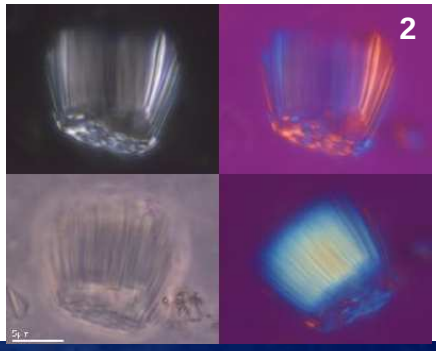
Plate 47. *Ceratolithaceae* Norris 1965
Scale bars = 5µm
1-3. *C cristatus* [HET] coccolithomorpha type; 31º04’ S – 16º28’ E (50m) South Atlantic [AMT16 cruise, May 2005]; XPL
4. *C cristatus* [HET] coccolithomorpha type; 25º96’ S – 21º56’ W (105m) South Atlantic [AMT16 cruise, May 2005]; XPL

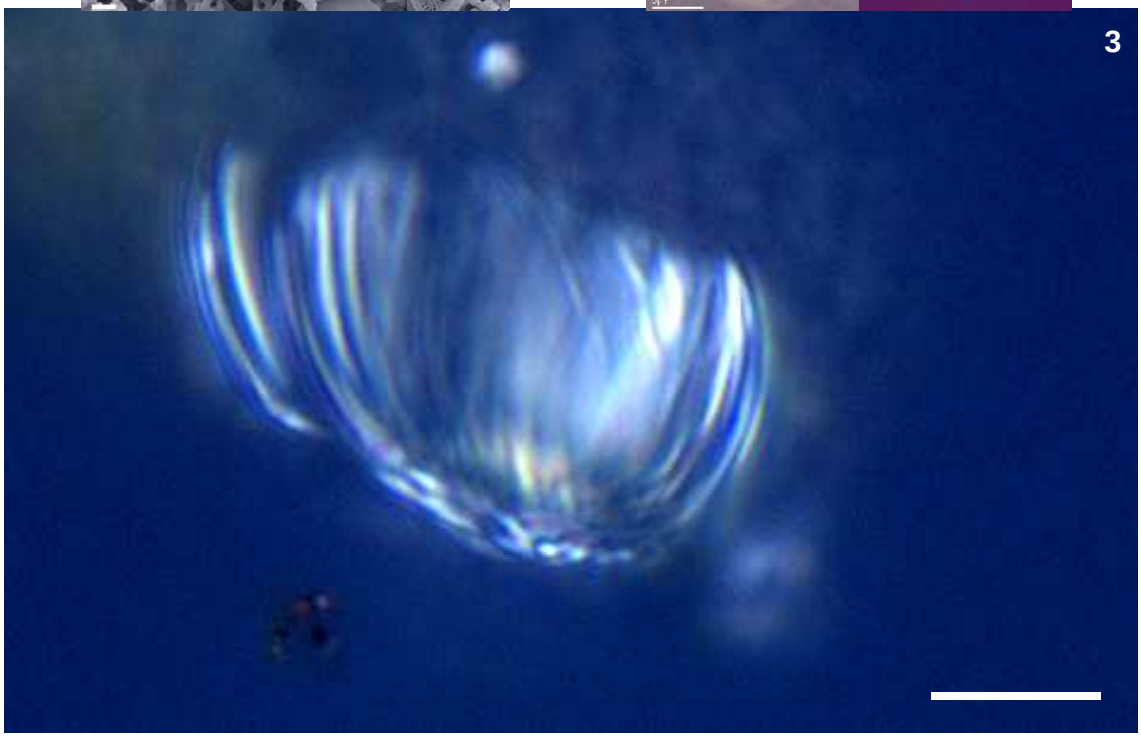


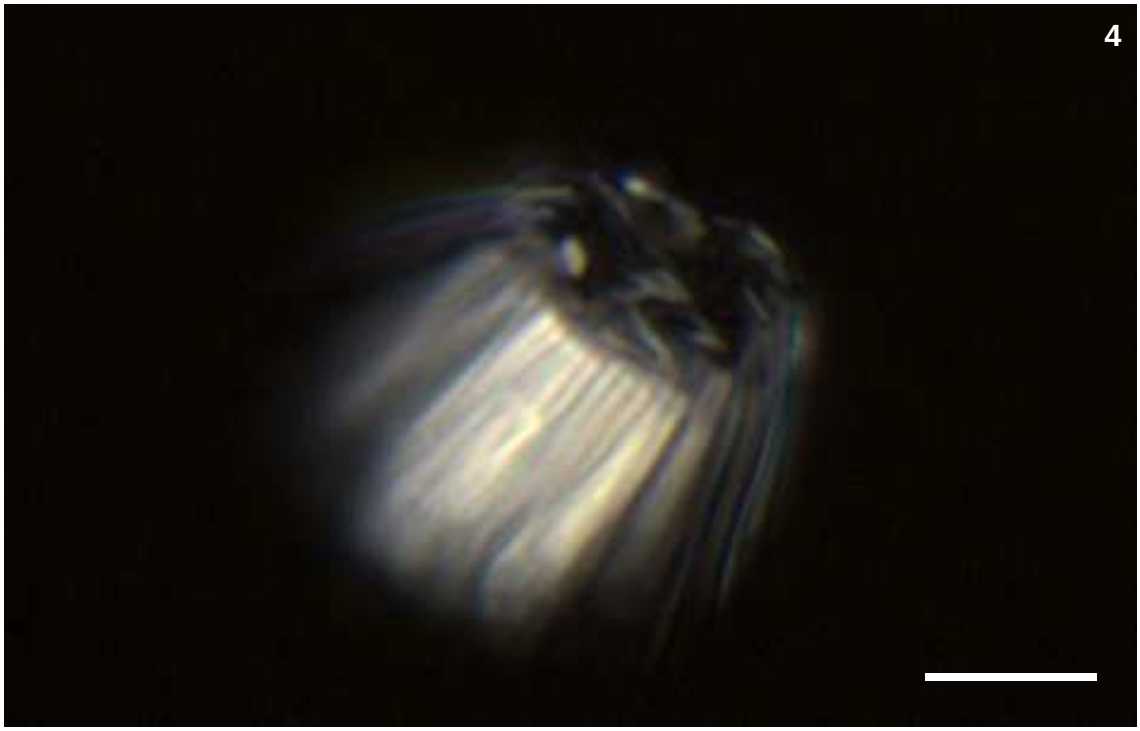
Incertis sedis

Plate 48. *Gladiolithus* Jordan & Chamberlain 1993 [*Thorosphaera*]
Scale bars = 5µm
1. *G flabellatus* [Halldal & Markali 1955] Jordan & Chamberlain 1993 [*Thorosphaera*] (Plankton*Net; Authors: Jeremy Young; Copyright: Jeremy Young, NHM); SEM
2. *G flabellatus*, cross-polarization image +/- gypsum plate and phase contrast (Plankton*Net; Authors: Jeremy Young; Copyright: Jeremy Young, NHM); SEM
3. *G flabellatus* [HET]; 31º49' S – 10º30' E (96m) South Atlantic [AMT16 cruise, June 2005]; XPL
4. *G flabellatus* [HET]; 31º58' S – 16º58' E (50m) South Atlantic [AMT16 cruise, June 2005]; XPL


1


2


3


4